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(54) Title: HISTAMINE AND SEROTONIN BINDING MOLECULES

(57) Abstract

The present invention relates to histamine and serotonin binding molecules that possess a binding site with the precise molecular configuration that is necessary to confer on the molecule a high affinity for histamine. The invention includes proteins, peptides and chemical compounds that possess this molecular configuration and that are thus able to bind to histamine with high affinity. These molecules may be used in the regulation of the action of histamine or serotonin, the detection and quantification or histamine or serotonin and in the treatment of various diseases and allergies. The molecules may also be used as components of vaccines directed against blood-sucking ectoparasites.

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Histamine and Serotonin binding molecules

The present invention relates to histamine and serotonin binding molecules. More particularly, the present invention relates to molecules possessing a binding site with the precise molecular configuration that is necessary to confer on the molecule a high affinity for histamine. Included as embodiments of the present invention are those proteins, peptides and chemical compounds that possess this molecular configuration and that are thus able to bind to histamine with high affinity. The molecules of the present invention may be used in the regulation of the action of histamine or serotonin and are thus useful in the detection and quantification of histamine or serotonin and in the treatment of various diseases and allergies.

Vasoactive amines such as histamine and serotonin are mediators of inflammation and regulators of certain physiological processes in animals, including humans. Histamine is present in the secretory granules of mast cells and basophils and is formed by decarboxylation of histidine. It is also present in ergot and plants and may be synthesised synthetically from histidine or citric acid.

The main actions of histamine in humans are stimulation of gastric secretion, contraction of most smooth muscle tissue, cardiac stimulation, vasodilation and increased vascular permeability. In addition to its regulatory role in immune reactions and inflammatory processes, histamine also modulates the production of many cytokines in the body (including those that regulate inflammation) and can interfere with the expression of cytokine receptors. Furthermore, histamine promotes wound healing.

The main pathophysiological roles of histamine are as a stimulant of gastric acid secretion and as a mediator of type I hypersensitivity reactions such as urticaria and hay fever. Histamine and its receptors also have pathological aspects to their functions. They play dominant roles in allergies such as asthma, allergic rhinitis, atopic dermatitis and food and drug allergies, which affect a great number of people and are an important cause of illness and mortality. Histamine or its receptors may also be involved either directly or indirectly in autoimmune disease, e.g. arthritis, and in tumour growth (Falus, 1994).

The hormone serotonin (also known as 5-hydroxytryptamine) is both a vasoconstrictor and a neurotransmitter. It can also increase vascular permeability, induce dilation of capillaries and cause the contraction of nonvascular smooth muscle. Serotonin is present in the brain

and intestinal tissues and is produced by the pineal gland and by blood platelets. Pathological aspects related to serotonin include abnormal blood pressure, migraine, psychological disorders, respiratory disease and coronary heart disease. Serotonin agonists and antagonists are used to treat some of these disorders, but again often have undesirable side-effects.

Anti–histamine drugs are widely used, especially for the treatment of allergies. Most of these drugs are compounds that are structurally related to histamine, and bind to its receptor(s), thereby obstructing the interaction of histamine with its receptor(s). Such drugs as are currently available often have undesirable side effects (for example drowsiness) and are not always effective.

Histamine produces its actions by an effect on specific histamine receptors which are of three main types, H_1, H_2 and H_3, distinguished by means of selective antagonist and agonist drugs. Histamine H_1 and H_2 antagonists have clinical uses but at present histamine H_3 antagonists are used mainly as research tools. Intracellular histamine appears to be involved in cellular growth (tumour growth promotion) and tissue repair. Currently undefined intracellular histamine receptors are thought to be involved in these processes (Falus, 1994).

Histamine receptors have been the subject of concentrated research for a number of years. However, scant information is available regarding the structure of the active site of these molecules - in fact the H_3 receptor has not yet been cloned. The lack of any direct structural information for these proteins is presumably due to the fact that histamine receptor proteins are membrane proteins that denature in the absence of lipid and are consequently very difficult to crystallise.

Based on the fact that the H_1 and H_2-type receptors belong to the broad class of seven transmembrane G protein-coupled receptors, it can be assumed that they are mainly alpha-helical. A number of site-directed mutagenesis studies have been performed on these receptors that have indicated certain residues that are important for histamine binding. In the H_2 receptor, Asp^{98}, Asp^{186} and Thr^{190} are believed to contribute to the histamine binding pocket (Gantz *et al.*, 1992).

Conventional H_1 receptor antagonists are widely used as antihistamines for treating allergic reactions including allergic rhinitis (hay fever), urticaria, insect bites and drug hypersensitivities. Drugs that lack sedative or muscarinic receptor antagonists are

preferred. H_1 receptor antagonists are also used as anti-emetics for the prevention of motion sickness or other causes of nausea including severe morning sickness. Muscarinic receptor antagonist actions of some antihistamines probably contribute to efficacy but also cause side-effects. Some H_1 receptor antagonists are fairly strong sedatives and may be used for this action.

However, there are numerous undesirable effects of the H_1 receptor antagonists currently used. When used for purely antihistamine actions, all of the CNS effects are unwanted. When used for their sedative or anti-emetic actions, some of the CNS effects such as dizziness, tinnitus and fatigue are unwanted. Excessive doses can cause excitation and may produce convulsions in children. The peripheral anti-muscarinic actions are always undesirable. The commonest of these is dryness of the mouth, but blurred vision, constipation and retention of urine can also occur. Unwanted effects not related to the drug's pharmaceutical action are also seen. Thus, gastrointestinal disturbances are fairly common while allergic dermatitis can follow topical application of these drugs.

H_2 antagonists are frequently used as inhibitors of gastric acid secretion. They are used as the drugs of choice in the treatment of peptic ulcer, as second line drugs in the treatment of Zollinger-Ellison syndrome and for treating reflux oesophagitis. Unwanted effects have been reported that include diarrhoea, dizziness, muscle pains, transient rashes and hypergastrinaemia. Some H_2 receptor antagonists can cause gynaecomastia in men and confusion in the elderly.

Besides these unwanted effects, some histamine antagonists are troublesome if taken with alcohol or with drugs. For example, the antihistamine Seldane used in combination with antibiotics and antifungals may cause life-threatening side-effects.

It can therefore be seen that drugs used to control the actions of histamine are not always effective. The reasons why they may have limited efficacy may relate to the specificity of these drugs for only a sub-class of histamine receptors, particularly when a certain class of conditions require interference with a larger class of receptors. Molecules that actually bind to histamine itself would compete for histamine binding with all receptors and may thus be more suitable for treating certain conditions.

There is thus a great need for effective antagonists of histamine and serotonin that do not generate the side-effects that detract from their applicability to the treatment of human and animal disorders.

There is also a great need for the quantification of histamine in, for example, food products, various bodily fluids (e.g. plasma or urine) or cell culture supernatants to monitor the effects of certain allergens, for example, or to indicate a potential specific antagonistic therapy for an allergic reaction. Currently-used systems (radioimmunoassays and ELISAs) utilise antibodies against histamine or against histamine derivatives. However, histamine is not very immunogenic, making it hard to raise high affinity antibodies against it, and most of the quantification systems that are currently used are not very sensitive or require modification of the histamine to be measured (for example by methylation or acylation). The use of molecules that bind to histamine in its natural form that would replace antibodies in assays like these would provide a highly sensitive system for the measurement of unmodified histamine. Similarly, molecules that bind to serotonin could be used for the quantification of this molecule.

Molecules capable of binding to histamine have previously been identified in blood-feeding ectoparasites. For example, a salivary nitric oxide–carrying haeme protein (nitrophorin) of the triatome bug *Rhodnius prolixus* has been found to bind histamine (Ribeiro and Walker, 1994). The isolation of four vasoactive amine binding proteins (VABPs) from ticks is described in co-pending International Patent Application No. PCT/GB97/01372 which is owned by the Applicant for the present invention. The contents of PCT/GB97/01372 are incorporated into the present application in their entirety.

These proteins bind to histamine and are closely related to one another. They are named MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6. Some of these molecules also bind serotonin (for example FS-HBP2). In other cases, such as in the case of D.RET6 for example, binding of serotonin is thought to alter the affinity of the molecule for histamine. The DNA sequences that encode these proteins are presently being used to isolate other related proteins in the same family from the same and different species.

These molecules appear to differ markedly from histamine binding proteins from any of the H_1, H_2 or H_3 families and appear to bind to histamine in a different manner. The elucidation of the structure of the histamine binding site of these molecules would markedly accelerate the rational design of effective histamine antagonists that would be unlikely to suffer from the side-effects which are associated with conventional anti-histamine agents.

Summary of the invention

According to a first aspect of the present invention there is provided a histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which has a binding site comprising amino acid residues phenylalanine, isoleucine or leucine at position I, tryptophan at position II and aspartate or glutamate at positions III and IV wherein residues I to IV are positioned substantially the same as residues 108, 42, 39 and 82 respectively in either of SEQ. ID. Nos 1 or 2, or residues 107, 41, 38 and 78 in SEQ ID 3 or residues 122, 54, 50 and 95 in SEQ ID 4, and functional equivalents thereof. Hereafter, this binding site will be referred to as the "first binding site". The proteins identified in SEQ IDs 1 to 4 are known as FS-HBP1, FS-HBP2, MS-HBP and D.RET6 respectively.

According to a second aspect of the present invention there is provided a histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which has a binding site comprising amino acid residues phenylalanine or isoleucine at residue I, tryptophan at residue II and aspartate or glutamate at residues III and IV wherein residues I to IV are positioned substantially the same as residues 98, 137, 24 and 120 respectively in either of SEQ. ID. Nos 1 or 2, or residues 95, 138, 23 and 120 in SEQ. ID. 3 or residues 112, 149, 35 and 135 in SEQ. ID. 4, and functional equivalents thereof. Hereafter, this binding site will be referred to as the "second binding site".

According to a third aspect of the invention there is provided a histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which comprises both the first and second binding sites described above, and functional equivalents thereof. For some histamine binding compounds containing both the first and second binding sites (such as D.RET6), binding of serotonin to the compound is thought to alter the affinity of the compound for histamine.

Other chemical compounds with a related action to serotonin may also influence the binding of histamine to a histamine binding compound containing both the first and the second binding sites. These related compounds include cysteinyl leukotrienes (such as leukotriene D_4 and leutkotriene E_4), platelet activating factor and thromboxanes.

According to a fourth aspect of the present invention there is provided a histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which comprises the sequence of D.RET6 (SEQ. ID. 4) or a fragment thereof and which comprises a first and second binding site as defined above, or functional equivalents thereof, wherein binding of serotonin to the compound increases the affinity of the compound for histamine.

By binding site is meant the specific region in the compound that contributes directly to the binding of a histamine or serotonin molecule. As such, binding at this site will comprise molecular recognition events between the binding site and the histamine or serotonin molecule, regulated by functional complementarities of shape, size, charges, H-bonds, hydrophobic and pi interactions and van der Waal's forces. Interactions may also comprise covalent chemical bonds.

By the term " functional equivalent" is meant compounds that possess the desired binding site and includes any macromolecule or molecular entity that binds to histamine or serotonin with a dissociation constant of 10^{-7}M or less and that possesses an equivalent complementarity of shape to that possessed by the binding sites of the histamine or serotonin binding molecules identified in any of SEQ IDs 1 to 4. A functionally equivalent complementarity of shape may be provided by any hydrogen, oxygen, phosphorus and nitrogen atoms that are positioned substantially as identified in the structures disclosed herein.

Current methods of generation of compounds with affinity for a molecule of interest have been until recently relatively primitive. The notion of combinatorial chemistry and the generation of combinatorial libraries has, however, developed at great speed and facilitated the rational design and improvement of molecules with desired properties. These techniques can be used to generate molecules possessing binding sites identical or similar to those of the histamine or serotonin binding sites identified herein.

Such compounds may be generated by rational design, using for example standard synthesis techniques in combination with molecular modelling and computer visualisation programs. Under these techniques, the "lead" compound with a similar framework to the histamine or serotonin binding site is optimised by combining a diversity of scaffolds and component substituents.

Alternatively, or as one step in the structure-guided design of a molecular entity, combinatorial chemistry may be used to generate or refine the structure of compounds that mimic the histamine or serotonin binding site of histamine or serotonin binding compounds by the production of congeneric combinatorial arrays around a framework scaffold. These steps might include standard peptide or organic molecule synthesis with a solid-phase split and recombine process or parallel combinatorial unit synthesis using either solid phase or solution techniques (see, for example Hogan, 1997 and the references cited therein).

Alternatively, or as a portion of a histamine or serotonin binding molecule of the present invention, functional equivalents may comprise fragments or variants of the proteins identified in Figures 1 to 4 or closely related proteins exhibiting significant sequence homology. By fragments is meant any portion of the entire protein sequence that retains the ability to bind to vasoactive amines with a dissociation constant of 10^{-7}M or less. Accordingly, fragments containing single or multiple amino acid deletions from either terminus of the protein or from internal stretches of the primary amino acid sequence form one aspect of the present invention. Variants may include, for example, mutants containing amino acid substitutions, insertions or deletions from the wild type sequence of Figures 1 to 4.

The man of skill in the art will understand that the residues that contribute to the binding of vasoactive amines in the four proteins explicitly identified herein are maintained in the relevant position for binding to histamine or serotonin through the framework structure of the protein. Thus, the framework residues of the proteins are responsible for the exact positioning of the binding amino acids.

Accordingly, it is contemplated that any molecular framework capable of retaining these amino acid side-chains in the necessary positions for binding to histamine or serotonin will be suitable for use in accordance with the present invention. Of particular suitability will be cyclic peptides held in a precise framework by their linking groups and bonds. The amino acid side chains may be held in a position substantially identical to their position in the histamine or serotonin binding site of native histamine or serotonin binding compounds. Preferably, the cyclic peptides comprise between 6 and 30 amino acids, preferably between 8 and 20 amino acids. Of particular suitability is the cyclic octapeptide Ala-Glu-Ala-Phe-Ala-Glu-Ala-Trp.

Biologically-active peptides with histamine or serotonin binding sites according to the present invention may be generated using phage libraries. Nucleic acids encoding amino acid residues identified as participants in the binding of histamine or serotonin, together with nucleic acid encoding the surrounding framework residues may be fused to give a polypeptide unit of between 10 and 1000 residues, preferably between 25 and 100 residues. By fusion of this nucleic acid fragment with that encoding a phage protein, for example pIII of the bacteriophage fd, the fusion molecule may be displayed on the surface of phage. Screening of the phage library with histamine or serotonin will then identify those clones of interest. These clones can then be subjected to iterative rounds of mutagenesis and screening to improve the affinity of the generated molecules for histamine or serotonin.

Residues with analogous physical properties to those that comprise the histamine or serotonin binding site may also form part of a molecule according to the present invention. For example, with respect to the protein FS-HBP2, either of the charged residues glutamate or aspartate may occupy position 39 and 82 in the sequence. At position 108 in the sequence, it is envisaged that any hydrophobic amino acid residue may occupy this site, provided that steric concerns are satisfied with respect to the molecular configuration of the binding site. Phenylalanine, isoleucine and leucine are preferable residues at this position. At position 42, tryptophan is preferred.

Additionally, at position 100 in the histamine binding compound sequence, it is preferred that a tyrosine residue is present. This molecule is thought to contribute to the stability of histamine in the binding site. Any molecular structure that retains this amino acid side-chain or an equivalent in this position forms an aspect of the present invention.

Due to variations in the length and sequence of the four proteins explicitly described herein, the method of numbering residues differs between proteins. However, it will be apparent from the alignment shown in Figure 22 which residues correspond to the residues numbered according to the sequence of FS-HBP2.

It is envisaged that proteins according to the present invention may be stabilised by the presence of disulphide bridges in the structure. For example, the cysteines found in positions 48, 169, 119 and 148 of FS-HBP2 are conserved in all four histamine binding proteins identified so far. Two disulphide bridges are formed in FS-HBP2, one between cysteines 48 and 169, the other between 148 and 119. Accordingly, for any protein

fragment designed to mimic the structure of the natural histamine or serotonin binding compound binding site, these cysteine residues may be present in the sequence so that one or both disulphide bridges form within the protein structure.

It is preferred that in addition to the high affinity with which the compounds of the present invention bind to histamine or serotonin, this binding phenomenon is also specific for histamine or serotonin. The advantages that this specificity confer on the compounds will be obvious to the man of skill in the art. For example, for use as a pharmaceutical or in the quantification of the histamine content of a solution, it is of the utmost importance that compounds other than histamine are not bound by the compounds of the present invention. In the case of a pharmaceutical, lack of specificity might lead to unwanted side-effects; used in the quantification of histamine, non-specificity would lead to misleading and inaccurate results.

According to a fifth aspect of the invention there is provided a protein comprising the amino acid sequence given in SEQ. ID. No. 5. This sequence encodes a salivary *Rhipicephalus appendiculatus* protein termed Ra-Res. Its primary sequence was inferred from a cDNA that was obtained by screening a *R. appendiculatus* salivary gland cDNA library with the antiserum from a guinea pig which had developed resistance against *R. appendiculatus* ticks.

According to a sixth aspect of the invention there is provided a protein comprising the amino acid sequence given in SEQ. ID. No. 6. This sequence codes for a protein termed Av-HBP from *Amblyomma variegatum* ticks. Preliminary results of histamine-binding studies suggest an equilibrium dissociation constant (Kd) of 7.3 nM.

According to a seventh aspect of the invention there is provided a protein comprising the amino acid sequence given in either SEQ. ID. No. 7 or SEQ. ID. No.8. These sequences encode proteins termed Ih/Bm-HBP1 and Ih/Bm-HBP2 respectively and were isolated from a mixed *Boophilus microplus/Ixodes hexagonus* cDNA expression library. The library was screened with probes constructed from RT-PCR products obtained from *Boophilus microplus /Ixodes hexagonus* salivary gland RNA, using degenerate primers of which the design was based on conserved domains within the *R. appendiculatus* HBPs (FS-HBP1, FS-HBP2. MS-HBP1 and D.RET6).

According to an eighth aspect of the invention there is provided a protein comprising the amino acid sequence given in SEQ.ID. No. 9, the amino acid sequence given in

SEQ.ID. No. 10, the amino acid sequence given in SEQ.ID. No. 11. These sequences encode proteins termed Ih/Bm-HBP3, Ih/Bm-HBP4, and Ih/Bm-HBP5 respectively. These sequences were also isolated from the mixed *Boophilus microplus/Ixodes hexagonus* cDNA expression library mentioned above. Their sequences show convincing sequence similarity with the *R. appendiculatus* HBPs (FS-HBP1, FS-HBP2. MS-HBP1 and D.RET6), but also contain extensive domains that are not present in the traditional sequences (see Figure 22).

As will be clear to the man of skill in the art, the invention includes functionally-equivalent derivatives and fragments of the protein sequences given in SEQ.ID. Nos. 6 to 11. "Functionally-equivalent" is used in this context to indicate derivatives and fragments that retain the capacity to bind to vasoactive amines or that contain epitopes which can be used in the development of vaccines of antibodies that target any one of the proteins Ra-Res, Av-HBP, Ih/Bm-HBP1, Ih/Bm-HBP2, Ih/Bm-HBP3, Ih/Bm-HBP4, or Ih/Bm-HBP5. The derivatives may be derived from the wild type sequences of these proteins by single or multiple amino acid substitutions, additions, insertion and/or deletions or by chemical modification of one or more of the amino acids, for instance by deglycosylation of glycosylated forms.

The invention also includes proteins in the same family as Ra-Res, Av-HBP, Ih/Bm-HBP1, Ih/Bm-HBP2, Ih/Bm-HBP3, Ih/Bm-HBP4, or Ih/Bm-HBP5. A protein is considered to belong to the same family as any one of these proteins if 40% or more of the amino acids in the sequence are conserved. For examples, proteins may be compared in this manner using GCG's pileup command (Program manual for the Wisconsin package, 1994; gap creating penalty = 2.50; gap extension penalty = 0.05).

The proteins of these aspects of the invention include natural biological variants or geographical variations within the species from which the proteins are derived.

For instance, a derivative may include an additional protein or polypeptide fused to one of these proteins at its amino- or carboxy- terminus, or added internally in the sequence. The purpose of the additional polypeptide may be to aid detection, expression of separation or purification of the protein or may be to lend additional properties to the protein as desired.

Synthetic molecules designed to mimic the tertiary structure or active site of the Ra-Res, Av-HBP, Ih/Bm-HBP1, Ih/Bm-HBP2, Ih/Bm-HBP3, Ih/Bm-HBP4, or Ih/Bm-

HBP5 proteins constitute a further aspect of the invention.

For many applications, compounds according to the present invention may be fused to an effector or reporter molecule such as a label, toxin or bioactive molecule. Such molecules may comprise an additional protein or polypeptide fused to the histamine or serotonin binding compound at its amino- or carboxy-terminus or added internally. The purpose of the additional polypeptide may be to aid detection, expression, separation or purification of the histamine or serotonin binding compound or may be to lend additional properties to the compound as desired.

Particularly suitable candidates for fusion will be reporter molecules such as luciferase, green fluorescent protein, or horse radish peroxidase. Labels of choice may be radiolabels or molecules that are detectable spectroscopically, for example fluorescent or phosphorescent chemical groups. Linker molecules such as streptavidin or biotin may also be used. Additionally, other peptides or polypeptides may be fused to a histamine or serotonin binding compound. Suitable peptides may be, for example, β-galactosidase, glutathione-S-transferase, luciferase, polyhistidine tags, secretion signal peptides, the Fc region of an antibody, the FLAG peptide, cellulose binding domains, calmodulin and the maltose binding protein. Antibodies or peptides used to target the histamine or serotonin binding compounds more efficiently towards a site of action (for example antibodies against membrane proteins of mast cells) may also be fused to the histamine or serotonin binding compounds.

These fusion molecules may be fused chemically, using methods such as chemical cross-linking. Suitable methods will be well known to those of skill in the art and may comprise for example, cross-linking of the thiol groups of cysteine residues or cross-linking using formaldehydes. Chemical cross-linking will in most instances be used to fuse non-protein compounds, such as cyclic peptides and labels.

When it is desired to fuse two protein molecules, the method of choice will often be to fuse the molecules genetically. In order to generate a recombinant fusion protein, the genes or gene portions that encode the proteins or protein fragments of interest are engineered so as to form one contiguous gene arranged so that the codons of the two gene sequences are transcribed in frame.

The compounds of the present invention may also comprise histamine or serotonin binding compounds bound to a support that can be used to remove, isolate or extract histamine or

serotonin from body tissues, blood or food products. The support may comprise any suitably inert material and includes gels, magnetic and other beads, microspheres, binding columns and resins.

If proteinaceous, the histamine or serotonin binding compound may be derived from any organism possessing a protein in the same family as the histamine or serotonin binding compounds identified to date. By protein family is meant a group of polypeptides that share a common function and exhibit common sequence homology between motifs present in the polypeptide sequences. By sequence homology is meant that the polypeptide sequences are related by divergence from a common ancestor.

Preferably, proteins or protein fragments are derived from blood-feeding ectoparasites, spiders, scorpions or snakes or other venomous animals. More preferably, the proteins or protein fragments are derived from ticks, most preferably Ixodid ticks such as, for example, *Rhipicephalus appendiculatus*.

Most preferably, proteinaceous compounds according to the present invention are derived from any one of the proteins FS-HBP1, FS-HBP2, MS-HBP1, D.RET6, Ra-Res, Av-HBP, Ih/Bm-HBP1, Ih/Bm-HBP2, Ih/Bm-HBP3, Ih/Bm-HBP4, or Ih/Bm-HBP5.

Protein or peptide compounds according to the invention will preferably be expressed in recombinant form by expression of the encoding DNA in an expression vector in a host cell. Such expression methods are well known to those of skill in the art and many are described in detail in *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover (IRL Press, 1995) or in *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press, 1995). Protein compounds may also be prepared using the known techniques of genetic engineering such as site-directed or random mutagenesis as described, for example, in *Molecular Cloning: a Laboratory Manual*: 2nd edition, (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press) or in *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993).

Suitable expression vectors can be chosen for the host of choice. The vector may contain a recombinant DNA molecule encoding compounds of the present invention operatively linked to an expression control sequence, or a recombinant DNA cloning vehicle or vector containing such a recombinant DNA molecule under the control of a promoter recognised by the host transcription machinery.

Suitable hosts include commonly used prokaryotic species, such as *E. coli*, or eukaryotic yeasts that can be made to express high levels of recombinant proteins and that can easily be grown in large quantities. Mammalian cell lines grown *in vitro* are also suitable, particularly when using virus-driven expression systems such as the baculovirus expression system which involves the use of insect cells as hosts. Compounds may also be expressed *in vivo*, for example in insect larvae or in mammalian tissues.

According to a ninth aspect of the present invention there is provided a pharmaceutical composition comprising a histamine or serotonin binding compound according to the first or second aspect of the invention or a protein according to the third, fourth, fifth, sixth, seventh or eighth aspects of the invention, in conjunction with a pharmaceutically-acceptable excipient. Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline (0.01M phosphate salts, 0.138M NaCl, 0.0027M KCl, pH7.4). Pharmaceutical compositions may also contain additional preservatives to ensure a long shelf life in storage.

According to a yet further aspect, the present invention provides for the use of the histamine or serotonin binding compounds of the first, second, third or fourth aspects of the invention, of a protein according to the fifth, sixth, seventh or eighth aspects of the invention or of the pharmaceutical compositions of the ninth aspect of the invention in therapy. The histamine or serotonin binding compounds, proteins or compositions may be used as anti-inflammatory agents or may be used to bind histamine or serotonin in mammals, thereby to regulate their action and to control their pathological effects. This causes their sequestration and so lowers the effective concentration of histamine or serotonin in the body. This results in a tempered or even entirely abrogated physiological response, depending upon the dosage used. The histamine or serotonin binding compounds of the present invention may also be used as anti–inflammatory agents or agents to counter the effects of allergic reactions in the body.

According to this aspect of the invention, the histamine or serotonin binding compounds, proteins or compositions may be used in conjunction with serotonin in order to alter the affinity of the compounds for histamine. For example, for the compound D.RET6 it is shown herein that serotonin significantly increases the affinity of the compound for histamine. Compounds related in action to serotonin may also be

used, such as cysteinyl leukotrienes (such as leukotriene D_4 or leutkotriene E_4), platelet activating factor, or thromboxanes.

The histamine or serotonin binding compound according to the first, second, third or fourth aspects of the invention or protein according to the fifth, sixth, seventh or eighth aspects of the invention may constitute the sole active component of the composition or can form part of a therapeutic package, such as a component of creams for topical administration to insect, snake or scorpion bites, or to skin affected by dermatitis. The proteins may also be used as carrier molecules for histamine or serotonin and histamine or serotonin–related compounds, in creams, oils, powders or pills, to provide slow release of the bound histamine or serotonin.

The invention also comprises the use of the compounds of the present invention as histamine or serotonin binding components in kits for the detection or quantification of histamine or serotonin levels (for example, in blood, nasal lavage fluid, tissues or food products). Such a kit will resemble a radio-immunoassay kit and would comprise a histamine or serotonin binding compound according to the present invention and detection means that allows the accurate quantification of the amount of histamine or serotonin in the fluid. A set amount of radiolabelled histamine or serotonin, for example tritiated histamine or tritiated serotonin, is added to the sample to be measured. The histamine or serotonin in the sample will then compete with the labelled histamine or serotonin for binding to the limited amount of binding sites possessed by the histamine or serotonin binding compounds also present in the sample. The amount of histamine or serotonin present in the sample can thus be accurately assessed.

One aspect of the present invention comprises such kits incorporating the histamine or serotonin binding compounds of the present invention. The histamine or serotonin binding compounds may be bound to magnetic beads, agarose beads or may be fixed to the bottom of a multiwell plate. This will allow the removal of the unbound labelled histamine or serotonin from the sample after incubation. Alternatively the protein may be bound to SPA (Scintillation Proximity Assay) beads, in which case there is no need to remove unbound ligand. Using a set of unlabelled histamine or serotonin standards, the results obtained with these standards can be compared with the results obtained with the sample to be measured.

The histamine or serotonin binding compounds of the first, second, third or fourth aspects of the invention, or proteins according to the fifth, sixth, seventh or eighth aspects of the invention can also be used for the detection of histamine or serotonin. Any technique common to the art may be used in such a detection method and may comprise the use of blotting techniques (Towbin *et al*, 1979), binding columns, gel retardation, chromatography, or any of the other suitable methods that are widely used in the art. In another embodiment, the histamine or serotonin binding compound may be fused either genetically or synthetically to another protein such as alkaline phosphatase, luciferase or peroxidase in order to facilitate its detection.

It may be preferred to include serotonin or a related compound in the kits according to this aspect of the invention in order to alter the affinity of the histamine binding compound for histamine. Such related compounds include cysteinyl leukotrienes (such as leukotriene D_4 or leukotriene E_4), platelet activating factor, and thromboxanes. This will be particularly preferred when the compound D.RET6 or a functional equivalent thereof is used as the active histamine binding compound of the kit.

The invention also comprises the use of the histamine or serotonin binding compounds of the first, second, third or fourth aspects of the invention, or proteins according to the fifth, sixth, seventh or eighth aspects of the invention as histamine or serotonin –binding entities bound to a support that can be used to remove, isolate or extract histamine or serotonin (from body tissues, blood or food products). The support may comprise any suitable material and includes gels, beads, microspheres, binding columns and resins. The histamine or serotonin binding compound can, for example, be chemically or enzymatically linked to reactive groups on these supports.

The present invention also includes the use of a histamine or serotonin binding compound of the first, second, third or fourth aspects of the invention, or of a protein according to the fifth, sixth, seventh or eighth aspects of the invention as tools in the study of inflammation, inflammation–related processes or other physiological effects of vasoactive amines such as the role of histamine in the formation of gastric ulcers. For example, the histamine or serotonin binding compounds may be used for histamine or serotonin depletion in cell cultures or in inflamed animal tissues, in order to study the importance of histamine or serotonin in these systems. The histamine or serotonin

binding compounds may be pre-incubated with serotonin, or a related compound to increase the affinity of the compounds for histamine.

The present invention also provides for the use of the histamine or serotonin binding compounds of the first, second, third or fourth aspects of the invention, and of a protein according to the fifth, sixth, seventh or eighth aspects of the invention as immunogens for use as metazoan parasite vaccines and in particular as protective immunogens in the control of diseases caused by arthropod and other metazoan parasites. Suitable candidates for vaccination include domesticated animals such as cattle, goats, sheep, dogs, cats and other animals that require protection against metazoan parasites, especially ticks. The vaccine may include adjuvants of the type which are well known in the art.

Nucleic acid molecules comprising a nucleotide sequence encoding a histamine or serotonin binding compound of the first, second, third or fourth aspects of the invention, or encoding a protein according to the fifth, sixth, seventh or eighth aspects of the invention form further aspects of the invention. These molecules include DNA, cDNA and RNA, as well as synthetic nucleic acid species.

Complementary DNAs encoding particular histamine or serotonin binding molecules according to the proteins FS-HBP1, FS-HBP2, MS-HBP1, D.RET6, Ra-Res, Av-HBP, Ih/Bm-HBP1, Ih/Bm-HBP2, Ih/Bm-HBP3, Ih/Bm-HBP4, or Ih/Bm-HBP5 are disclosed herein in Figures 1 to 11 (nucleotides and amino acids are given in their standard one letter abbreviations).

The preferred nucleic acid molecule, according to the invention, comprises a nucleotide fragment identical to or complementary to any portion of any one of the nucleotide sequences shown in Figures 1 to 11 that encodes a histamine or serotonin binding compound, or a sequence which is degenerate or substantially homologous therewith, or which hybridises with the said sequence. By 'substantially homologous' is meant sequences displaying at least 60% sequence homology. 'Hybridising sequences' included within the scope of the invention are those binding under standard non–stringent conditions (6 X SSC/50% formamide at room temperature) and washed under conditions of low stringency (2 x SSC, room temperature, or 2 x SSC, 42°C) or preferably under standard conditions of higher stringency, e.g. 0.1 x SSC, 65°C (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

The nucleic acid sequences according to the invention may be single- or double–stranded DNA, cDNA or RNA. Preferably, the nucleic acid sequences comprise DNA.

The invention also includes cloning and expression vectors containing the DNA sequences of the invention. Such expression vectors will incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter–operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the invention.

Additionally, in the absence of a naturally-effective signal peptide in the protein sequence, it may be convenient to cause the recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses). Many such vectors and expression systems are well known and documented in the art. Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

The expression of heterologous polypeptides and polypeptide fragments in prokaryotic cells such as *E. coli* is well established in the art; see for example *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press or *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover (IRL Press, 1995). Expression in eukaryotic cells in culture is also an option available to those skilled in the art for the production of heterologous proteins; see for example O'Reilly *et al.*, (1994) *Baculovirus expression vectors - a laboratory manual* (Oxford University Press) or *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press, 1995) .

Suitable vectors can be chosen or constructed for expression of histamine or serotonin binding proteins, containing the appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. bacteriophage, or phagemid, as appropriate. For further details see *Molecular Cloning: a Laboratory Manual*. Many known techniques and protocols for manipulation of nucleic acid, for example, in the preparation of nucleic acid constructs, mutagenesis,

sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., (John Wiley & Sons, 1992) or *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993). For example, in eukaryotic cells, the vectors of choice are virus-based.

A further aspect of the present invention provides a host cell containing a nucleic acid encoding a histamine or serotonin binding compound of the first, second, third or fourth aspects of the invention, or encoding a protein according to the fifth, sixth, seventh or eighth aspects of the invention. A still further aspect provides a method comprising introducing such nucleic acid into a host cell or organism.

Introduction of nucleic acid may employ any available technique. In eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection or transduction using retrovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage.

Introduction of the nucleic acid may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

Transgenic animals transformed so as to express or overexpress in the germ line one or more histamine or serotonin binding compounds as described herein form a still further aspect of the invention, along with methods for their production. Many techniques now exist to introduce transgenes into the embryo or germ line of an organism, such as for example, illustrated in Watson *et al.*, (1994) Recombinant DNA (2nd edition), Scientific American Books.

A variety of techniques are known and may be used to introduce the vectors according to the present invention into prokaryotic or eukaryotic cells. Suitable transformation or transfection techniques are well described in the literature *Molecular Cloning: a*

Laboratory Manual: 2nd edition, (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press). In eukaryotic cells, expression systems may either be transient (e.g. episomal) or permanent (chromosomal integration) according to the needs of the system. See, for example *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., (John Wiley & Sons, 1992).

All documents mentioned in the text are incorporated herein by reference.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to proteinaceous histamine or serotonin binding compounds isolated from ticks. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of Figures

Figure 1 is the sequence of FS-HBP1 (SEQ. ID. 1), showing sequencing primers and sequencing strategy.

Figure 2 is the sequence of FS-HBP2 (SEQ ID 2), showing sequencing primers and sequencing strategy.

Figure 3 is the sequence of MS-HBP1 (SEQ ID 3), showing sequencing primers and sequencing strategy.

Figure 4 is the sequence of D.RET6 (SEQ ID 4), showing sequencing primers and sequencing strategy.

Figure 5 is the sequence of ra-RES (SEQ. ID. 5). Asparagines that are part of putative glycosylation recognition sites are underlined and shown in italics. The squiggly line denotes a possible amidation site I; the double line indicates the putative polyadenylation signal and the polyA-tail is shown in bold letter type.

Figure 6 is the sequence of Av-HBP (SEQ. ID. 6). Sequence of the Av-HBP cDNA and its inferred primary structure. The cDNA has a remarkably long non-coding region, downstream of the stop codon.

Figure 7 is the sequence of Ih/Bm-HBP1 (SEQ. ID. 7).

Figure 8 is the sequence of Ih/Bm-HBP2 (SEQ. ID. 8).

Figure 9 is the sequence of Ih/Bm-HBP3 (SEQ. ID. 9).

Figure 10 is the sequence of Ih/Bm-HBP4 (SEQ. ID. 10).

Figure 11 is the sequence of Ih/Bm-HBP5 (SEQ. ID. 11).

Figure 12 is a silver-stained 12% SDS-polyacrylamide gel showing fractions obtained from a nickel resin column used to purify recombinant D.RET6 expressed in insect cells. A, flow-through fraction; B, first wash fraction (10 volumes of phosphate buffer pH 6.5); C, second wash fraction (with 5mM imidazole); D, third wash fraction (with 10mM imidazole); E, fourth wash fraction (with 15 mM imidazole); F, fifth wash fraction (with 20 mM imidazole); G, elution fraction with 300mM Imidazole: H, purified D.RET6 after ion exchange chromatography.

Figure 13 is a silver-stained 12% SDS-polyacrylamide gel of D.RET6 overexpressed in insect cells. Lanes A and B shows purified D.RET6 as a monomer (25 kDa), dimer (50 kDa) and trimer (85 kDa) and lane C is after deglycosylation. Cleaved oligosaccharides were retained in the stacking gel and can be seen at the top of lane C. Excess PNGase-F appears as a band of approximately 30 kDa. Lane D shows the purified monomeric form of D.RET6.

Figure 14 shows Western blot detection of native histamine-binding protein in *Dermacentor reticulatus* salivary gland extract. Lane A, non-reduced form; lane B, reduced form; lane C, deglycosylated form.

Figure 15: Determination of the IC_{50} of three unlabeled competitors e.g. histamine, 1-methylhistamine, and 3-methylhistamine by generating a competitive binding curve of recombinant D.RET6. The graphically derived of IC_{50} of the unlabelled ligands above in displacing the radioactive histamine from the binding site by 50% are 133 nM, 750 μM and 365μM, respectively.

Figure 16 shows saturation curves of histamine binding in the absence (solid line) or presence (dashed lines) of serotonin. ♦no serotonin, ■2.4 μM and Δ 23.8 μM serotonin, respectively.

Figure 17: Radioactive histamine-binding assay of the recombinant D.RET6 in the absence (A) and presence of serotonin at 2.4 μM (B) and 23.8 μM (C).

Figure 18: Depiction of Histamine bound in the binding pocket of the histamine binding site of a cyclic peptide.

Figure 19: Three-dimensional depiction of Histamine bound in the binding pocket of the histamine binding site of a cyclic peptide.

Figure 20: (a) Ribbon diagram showing the arrangement of molecules A and B in the asymmetric unit of FS-HBP2. The 13 sheet is labelled A - I and α helices 1-3. The histamine ligands are shown as ball-and-stick representation, along with Tyr100. The arrow indicates the direction of view in (b) and the boxed region shows the portion enlarged in (c). (b) This view shows the helix and extended loop which occlude the barrel entrance. (c) Stereo view of the individual hydrogen bond contacts between residues in the A and B molecules. (d) Superposed C-α traces of molecules A and B [performed using SHP, (Stuart *et al.* 1979)].

Figure 21: Stereo views of the *H* (a), *L* (b) and *apo-L* (c) histamine binding sites, respectively. In each case the 2|Fo|-|Fc| map is displayed around the ligand. The figure shows the core structure of the protein (tube); contacting residues and secondary structure elements are labelled. In (c) the bound structure L (drawn transparent), has been superposed onto the apo coordinates.

Figure 22: An alignment of the cDNA-inferred amino acid sequences of the various HBPs, created using the pileup commands of the Genetics Computer Group. (1994). Program Manual for the Wisconsin Package, version 8. (575 Science Drive, Madison, Wisconsin, USA 53711).

EXAMPLES

Ticks

Ticks were reared according to Jones *et al.* (1988), as described in detail in co-pending International patent application PCT/GB97/01372, the contents of which are incorporated herein in their entirety.

The identification of proteins FS-HBP1, FS-HBP2, MS-HBP1 and D.RET6, and cloning of the encoding genes is also described in PCT/GB97/01372 (see Examples 1, 2, and 3).

The Ra-d0 (*Rhipicephalus appendiculatus*) library was constructed with mRNA from salivary glands of unfed *R. appendiculatus* ticks (127 males, 124 females). The Av- library (*Amblyomma variegatum*) was constructed with mRNA isolated from salivary glands of partially fed, adult *A. variegatum* ticks (50 males, fed for 6-7 days, and 50

females, fed for 4 to 5 days). The Ih/Bm library (a mixed *Ixodes hexagonus* and *Boophilus microplus* library) was constructed with the pooled mRNA from salivary glands of partially fed *Boophilus microplus* (30 females) and three-day fed *Ixodes hexagonus* ticks (20 males/20 females).

Example 1: Cloning and sequencing of the *Amblyomma variegatum*, *Ixodes hexagonus* and *Boophilus microplus* libraries

Using the RNAce Total Pure extraction kit (Bioline Ltd., UK), total RNA was isolated from salivary glands of partially fed *Amblyomma variegatum*, *Ixodes hexagonus* and *Boophilus microplus* ticks. The RNA samples were submitted to reverse-transcriptase polymerase chain reactions (RT-PCR), using the Titan One-Tube RT-PCR system (Boehringer Mannheim) and degenerate primers:

5'-AAYGGNGARCAYCARGAYGCNTGGAA; and

5'-KTRTMRTCNGTNRYCCANARYTCRTA, the design of which was based on conserved domains in the *Rhipicephalus appendiculatus* HBPs.

Cycling conditions were according to the manufacturer's suggestions, but a 48 °C annealing temperature was used. RT-PCR products were ligated into the pGEM-T vector (Promega), and partially sequenced, using the SP6 and T7 primer sites flanking the cloning site. Inserts of which the inferred amino-acid sequences showed similarity to the original *Rhipicephalus* HBPs were labeled with digoxygenin, using the High Prime DNA labeling kit (Boehringer Mannheim), and were used as probes to screen the libraries. An anti-digoxigenin antibody conjugated with alkaline-phosphatase was used to visualize probe hybridization. The Ra-d0 library was screened (according to Mierendorf et al.,1987) with serum from a guinea pig that had acquired resistance against *R. appendiculatus* ticks following repeated infestations.

The pBluescript SK (-) phagemids of positive clones were excised *in vivo*, using the R408 helper phage, as described by Short et al. (1988).

Example 2: Recombinant protein expression

Expression and purification of FS-HBP1, FS-HBP2, MS-HBP1 is described in detail in Example 3 of PCT/B97/01372.

1) Construction of clones for D.RET6

Using an *E. coli*-based expression system, the DNA sequence encoding D.RET6 (from Glu29 to Leu109) was subcloned as a *BclI/ XhoI* fragment into *BamHI /XhoI* -digested pET-23 a (+) in the same reading frame as the 6x His tag using the PCR technique with the following primers, 5'-TATATGATCAGAAAACCCGCTCTGGG-3' and 5'TATA CTCGAGCCA GGGTTCGCCGT-3' (the enzyme recognition sites are underlined.). The recombinant plasmid was transformed into host strain AD494(DE3) pLysS, which uses the T7 system, and success of the procedure was confirmed by sequencing.

D.RET6 was also expressed in bacteria. The bacterial transformant was grown at 37°C in Luria-Bertani medium containing ampicillin and chloramphenicol. The culture was induced at its exponential growth phase (OD_{600} about 0.5) using 0.5-1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and grown further for at least 4 hours before harvesting by centrifugation (4500xg, 4 °C, 5 mins.). The pellet was resuspended in the lysis buffer containing 6M urea, 20mM Tris pH8 and 500mM NaCl, sonicated briefly on ice, a few drops of Triton-X 100 added, and mixed by rolling at 4 °C for 5 mins. The supernatant was collected by centrifugation at 7500xg 4 °C for 30 mins.

D.RET6 was also expressed in the baculovirus expression system. The DNA fragment containing the complete coding sequence of D.RET6 was amplified using the oligonucleotides: 5'-TATGAAGATGCAGGTAGTGC-3' and:

5'-ATATGATCAGCCAGGGTTCGCCGT-3'.

The resulting PCR product was blunt-ended, digested with *BclI*, and ligated with the transfer vector pAcCL 29-1 -6xHis (Livingstone, 1989), which was prepared to have a blunt end at a *Sac* I site and an adhesive end at a *BamHI* site. The ligation product was then transferred into *E. coli* (XL1-Blue) and the transformant was grown to produce the plasmid. The plasmid was checked for the absence of any undesired mutations by complete resequencing. Sf9 cells were cotransfected at different ratios with the recombinant transfer vector and *Bsu36*I-cut *Autographa californica* polyhedrosis virus (BacPAK6) DNA in the presence of 8 μg of Lipofectin (Gibco BRL) per 24 μl reaction.

For expression, recombinant baculoviruses were identified as galactosidase-negative plaques by plating under Seaplaque agar. The putative recombinant clones were plaque purified once more. The clone from an infected Sf21 cell lysate that gave a positive result on a western blot using polyclonal anti-bacterial expressed D.RET6 antisera was

amplified and used in the production of the recombinant protein. For each subsequent production of the fusion protein, Sf9 cells were infected with these baculoviruses at MOI of about 5 and grown for 2-3 days before collecting the supernatant by centrifugation at 1000xg for 5 min. After 60% ammonium sulphate precipitation of the supernatant, the pellet was discarded. The pellet obtained at 100% ammonium sulphate precipitation was redissolved in Buffer A (50mM sodium phosphate, 300mM NaCl and 10% glycerol, pH8).

2) Protein purification and production of antisera

The purification steps were the same for both bacterial and baculovirus expression systems. Briefly, Ni^{2+}-nitrilo-triacetic acid resin, previously equilibrated in buffer A, was added to the supernatant or the solution. After incubation on a roller (2 hr 4 °C), the resin was transferred to a column and washed with 20 volumes of phosphate buffer (50mM sodium phosphate, 300mM NaCl and 10% glycerol, pH6.5). Protein was eluted with 5 volumes of 300mM Imidazole pH 8, concentrated, and the solvent replaced with phosphate-buffered saline using a Centricon-10 (Amicon) filtration unit. The recombinant D.RET6 was further purified on a HiTrap SP column (Pharmacia) using a gradient of 0.1-0.6 M NaCl in 50 mM MES pH 6.2.

To determine the location and solubility of the expressed protein in *E. coli*, the culture of the bacterial transformant was sampled before and 4 hours after IPTG-induction and processed according to the protocol described in the Qiaexpressionist booklet (QIAGEN). In brief, the sample was spun down, lysed by a cycle of freezing and thawing, and brief sonication, then centrifuged to separate insoluble proteins and the cytosolic soluble protein fraction. In another sampled culture, cells was pelleted and subjected to osmotic shock by, first, resuspending in 30mM Tris solution containing 20% sucrose (pH8) and incubating at room temperature with shaking in the presence of 1mM EDTA. The cells were then collected and resuspended in the low-osmotic ice-cold solution (5mM $MgSO_4$). The proteins released in the solution were collected by centrifugation. All fractions together with the pre-induced fraction were analysed by 12% SDS-PAGE.

Preparation of fusion proteins for antibody production and the technique for immunizing with small amounts of antigen are described by Sambrook *et al*, 1989. Briefly, guinea-pig anti-D.RET6 antiserum was prepared by repeated (x3)

intraperitoneal immunization of guinea pigs with homogenized 10% SDS-PAGE gel slices containing microgram quantities of bacterially-expressed purified D.RET6 in phosphate-buffered saline.

3) Electrophoresis and Western Blotting

SDS-PAGE gels and Western blots showing expression of FS-HBP1, FS-HBP2, MS-HBP1 is demonstrated in PCT/GB97/01372(see Figures 6 and 7).

Salivary glands (and other tissues) were excised from ticks at different time points of the feeding period, and homogenised in PBS. The homogenates were centrifuged at 10,000g for 5 minutes and the supernatants were submitted to sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE; Laemmli, 1970).

Figures 8 and 9 show the expression of D.RET6 in *E. coli* and insect cells, respectively.

For Western blotting, proteins were transferred to nitrocellulose (Gelman Sciences) by means of semi-dry electroblotting (Kyhse-Anderson, 1984) using an AE-6675 Horizblot MS-HBP1 from the first day p.a. until the end of the feeding period.

Figure 14 shows a Western blot showing expression of the D.RET6 protein.

4) Av-HBP protein expression and purification

The coding region of the Av-HBP cDNA was PCR amplified (95 °C for 30", 50 °C for 30", 72 °C for 30"; 18 cycles) using a forward primer designed to add a *Sac* I site upstream of the start codon (5'- TATGAGCTCATGAACTCTGCCTTGTGG; the *Sac*I site is underlined), and a reverse primer (5'- TATGGATCCGGGGTGGCCTCACCG) containing a *Bam*HI site (underlined). The product was ligated in between the *Sac* I and *Bam*HI sites of the pAcCl29.1 transfer vector (Livingstone and Jones, 1989) which had been modified by the insertion of six histidine codons and a stop codon, downstream of the *Bam*HI recognition site (see original patent). This resulted in the addition of the sequence Ile-$(His)_6$ to the carboxy- terminus of Av-HBP.

Co-transfection of Sf21 Spodoptera cells with the transfer vectors and baculovirus (BacPak6), and amplification of recombinant virus, were according to Kitts and Possee (1993). *Trichoplusia ni* cells (High Five, Invitrogen) and TC100 medium containing 10% foetal bovine serum (Gibco BRL) were used for protein expression. After 60 hours incubation at 28 °C, the secreted protein was precipitated from the medium with

ammonium sulphate (in the 45-80 % saturation fraction) and redissolved in a 50 mM sodium phosphate buffer (pH 8.0), containing 300 mM NaCl and 10% glycerol (buffer A). Talon beads (Clontech) were added to capture the oligohistidine-tagged proteins (1 hour incubation at 4°C). The beads were applied to a 10 ml-column, and washed with buffer A (20 volumes), then with 50 mM sodium phosphate buffer (pH 7.0), containing 300 mM NaCl and 10% glycerol (20 volumes), and finally with 50 mM sodium phosphate buffer (pH 7.0; 20 volumes). The protein was eluted using 200 mM imidazole in sodium phosphate buffer (100 mM, pH adjusted to 8.0).

Example 3: Characterisation of proteins

1) Histamine binding assays

The purified recombinant proteins were submitted to histamine binding assays as set out in Warlow and Bernard (1987). This method used protein precipitation to separate free from bound ligand (radiolabelled histamine) by addition of polyethylene glycol (molecular weight 8000) and centrifugation. In all experiments, thin-layer chromatographs were run in an acetate-ammonia solvent system after a four hour incubation period to ensure that no metabolisation of histamine had occurred.

Saturable binding of ^{3}H-histamine was obtained with FS-HBP1, FS-HBP2, MS-HBP1 rHBPs (see Figure 20 of PCT/GB97/01372).

For Av-HBP, semi-purified recombinant protein was incubated with varying amounts of tritiated histamine, and the amount of bound radiolabel was determined by liquid scintillation counting. Separation of free from bound ligand was obtained following the method described by Warlow and Bernard (1987), which uses polyethylene glycol (PEG 8000) to precipitate the protein. Results (not shown) suggested that the equilibrium dissociation constant (Kd) for histamine is around 7.3nM.

2) Radioligand binding assay for D.RET6

The recombinant D.RET6 was diluted with 1.5% γ-globulin (Sigma) and used in one set of experiments. Fifty microlitres of the protein solution were incubated with 50 µl of 1:2500 dilution of [2,5-^{3}H] histamine diHCl (1µCi/µl) solution (Amersham) at room temperature for at least 3 hours with or without increasing concentrations of unlabelled histamine. All the assays were carried out in a total volume of 200 µl. The incubations were terminated by adding 125 µl of PEG 8000 (36% w/v in PBS) and centrifuged in a

microfuge at maximum speed for 12 minutes to collect the bound protein. The tubes were spun once more to remove all supernatant without disturbing the pellets. Subsequently, pellets were redissolved in PBS. Three millilitres of liquid scintillation cocktail (Beckman) were added and the radioactivity measured using a liquid scintillation counter (Wallac, 1217 Rackbeta).

In a second set of experiments, a competitive binding assay was used to compare three unlabelled competitor ligands (histamine, 1-methylhistamine and 3-methylhistamine).

To study the effect of serotonin on histamine binding activity, 10 µl of PBS (as a control experiment) or 10 µl serotonin (50 µM or 500 µM) were added to each 200 µl binding assay.

For data analysis, the ligand affinity constant was estimated from Scatchard plots as previously described by Hulme, 1992, *Receptor-Ligand Interactions, IRL Press, Oxford*). The nonlinear regression was used to fit the data (Motulsky, 1987, *FASEB J*, **1**: 365-374) and two asymtotic straight lines were made as described elsewhere (Feldman, 1972, *Analytical Biochem* **48**: 317).

From the plotted curved line, two asymtotic straight lines were drawn according to Feldman (1972) consistent with two histamine binding sites of approximate Kd 6 x 10^{-8} M and 2 x 10^{-6} M. Comparison of the ability of histamine and its methylderivatives to displace radioactive histamine indicated that the binding by D.RET6 was specific for histamine (Figure 15). Surprisingly, the saturation curves and corresponding Scatchard plots for histamine binding in the presence of serotonin revealed a marked synergistic effect (Figures 15 and 16). At a final concentration of 2.38µM serotonin, the Kd for the two binding sites for histamine was 1.1 x 10^{-9} M and 1 x 10^{-6} M, and with 23.8µM serotonin, 1.3 x 10^{-9} M and 1 x 10^{-6} M, respectively.

Thus, in the presence of serotonin, the binding affinity of D.RET6 for its ligand, histamine, was found to increase sixty-fold. Recently, it has been reported that external stimuli (including serotonin) regulate mammalian H1 receptor activity, provoking increased ligand affinity (Bloemers, S. M., Verheule, S., Peppelenbosch, M. P., Smit, M. J., Tertoolen, L. G.J., and De Laat, S. (1998) *The Journal of Biological Chemistry* **273**(4), 2249-2255). Although the molecular details remain unclear, a conformational change in the H1 receptor, induced by serotonin, has been proposed to explain the

increase in affinity for histamine (Bloemers, 1998). It thus seems possible that the synergistic effect of serotonin on the tick histamine-binding protein has evolved to counteract serotonin-induced enhanced binding affinity of H1 receptors. Such a mechanism might therefore enable the tick protein to outcompete the host's serotonin-sensitized histamine receptors in the feeding site. As adult *D. reticulatus* feed on a variety of domestic and wild mammals, including dog, horse, cattle, sheep, deer, fox, hare and hedgehog, the synergistic effect of serotonin may provide flexibility in the performance of the tick's histamine-binding protein under a range of host-specific haemostatic responses.

This finding has important implications for the design of molecules with histamine binding activity and gives important insights into the mechanism of action of these tick proteins, along with molecules designed to mimic their action. For example, in order to alter the affinity of these molecules for histamine, serotonin may be delivered simultaneously in an appropriate amount.

Example 4: Crystallisation of proteins

Purified FS-HBP2 was dialysed against 10 mM histamine in water (the pH of the histamine solution was adjusted to 6.8 using NaOH), and concentrated using Centricon 10 centrifugation units (Amicon) to a final protein concentration of 20μg/μl. A hanging drop of 3μl of the protein/histamine solution combined with 2μl of mother liquor was allowed to equilibrate at room temperature with 1ml of mother liquor [0.1 M MES buffer (pH 6.5), containing 0.01 M cobalt chloride hexahydrate and 1.8 M ammonium sulphate (Hampton Research)].

Native data to 2.24Å resolution and derivative data to 3 Å were collected at room temperature using in-house Mar-research imaging plate detectors attached to Rigaku rotating anode generators. Frozen native and semi-apo data were collected (to 1 .24 Å and 1 .35 Å resolution respectively) at the Brookhaven National Laboratory synchrotron using a Brandeis single module CCD detector (see Table 1). The semi-apo crystal was prepared by immersion in several changes of histamine-free mother liquor during the week prior to data collection. Mother liquor containing 30% glycerol was used as a freezing solution. Crystals were kept at 100K using a cryostream (Oxford Cryosystems). Freezing resulted in a distinct change in unit cell dimensions, with axes b and c smaller

and axis a slightly larger than at room temperature. Diffraction data were processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997).

Phasing and model building

The structure was solved by MIR using cis-platinum and trimethyllead-acetate (Holden and Rayment, 1991) derivatives (Table 1). Initially, 10-15° of data were collected for each new derivative. Data collection was continued only for crystals that showed appreciable isomorphous differences, otherwise they were transferred back to the mother liquor for the next soaking experiment.

Difference Patterson maps allowed two binding sites to be located, by inspection, for each heavy metal. Further sites and the common origin for the two derivatives were found by difference Fourier techniques. Trimethyllead-acetate introduces some degree of non-isomorphism (see Table 1). Data reduction, scaling and calculation of isomorphous differences were carried out with the in-house programs 3DSCALE and DIFFER (Stuart *et al.*, 1979). MIR-phases were calculated with MLPHARE (Otwinowski, 1991), and improved by solvent flattening with the program GAP (Grimes and Stuart, unpublished).

The initial maps revealed the presence of two molecules in the asymmetric unit. The noncrystallographic symmetry operator relating these molecules was determined and refined with GAP starting from the coordinates of the heavy metal binding sites and chosen marker positions from the electron density map. Non-crystallographic symmetry averaging resulted in an electron density map of high quality, which could be readily interpreted. Electron density map interpretation used FRODO (Jones, 1985) and 0 (Jones *et al.*, 1991).

TABLE 1

	Unit Cell (a,b,c)	Reso-lution (Å)	Total # reflect-ions	# Unique reflect-ions	Comp-leteness (%)	R-merge	% iso-morphous difference	# sites	Phasing power
Native 1	77.0,77.8,80.1	20-2.27	127,028	22,222	94.8	5.2	-	-	-
Cis Pt	76.9,77.8,80.1	20-3	35,045	9,898	97.6	8.0	20.5	6	1.6/1.3
TML	76.2,78.9,80.1	20-3	41,086	9,938	97.1	7.7	32.6	2	1.0/0.8

(a) Structure determination and refinement

	Native2	Apo
Unit cell (a,b,c)	77.5, 74.8, 78.6	77.5, 74.4, 77.9
Resolution range (Å)	20.0-1.24	20 – 1.35
No.of reflections	108,735	76,301
R-factor	18.4%	18.7%
No.of protein atoms	2744	2744
No.of water molecules	529	537
RMS bond length deviation	0.013Å	0.014Å
RMS bond angle deviation	1.5°	1.6°
Mean B-factor (Protein:A&B)	11Å^2	20^2
Mean B-factor (ligand, site *H*)	5Å^2	17Å^2
Mean B-factor (ligand, site *L*)	5Å^2	§
Mean B-factor (water)	26Å^2	36Å^2

(b) Refinement Statistics

Refinement and analysis

The initial map immediately revealed that FS-HBP2 had lipocalin topology. The coordinates for retinoic acid binding protein (IRBP) were used as an initial framework for model building. The initial model was refined against the room temperature dataset (Nativel, Table 1) using XPLOR (Brtinger, 1992) (rigid body, positional and B-factor refinement). The resulting model was refined against the higher resolution (Native2) data. Rigid body refinement yielded improved NCS operators which allowed positional B-factor and positional refinement to 1.24Å resolution. Further refinement included overall anisotropic B-factor refinement and simulated annealing. Water and histamine molecules were then included along with a bulk solvent correction. During the course of the refinement stereochemical restraints were modified so that eventually electrostatic and van der Waals terms were omitted from the target function.

PROCHECK (Laskowski *et al.*, 1993) was used for structure validation. Secondary structure assignments used DSSP (Kabsch and Sander, 1983). Structural superpositions

used SHP (Stuart *et al.*, 1979). Atomic coordinates used for comparisons were obtained from Protein Data Bank, Brookhaven National Laboratory.

Structure Determination of rFS-HBP2

The FS-HBP2-histamine complex crystallizes in an orthorhombic space group ($P2_12_12_1$) with 2 molecules per asymmetric unit. The unit cell dimensions vary, especially upon freezing (Table I). The structure was solved by the use of two heavy atom derivatives and refined using XPLOR (Brunger, 1992) to yield a final model with reasonable stereochemistry (RMS bond deviation 0.013 Å) and R-factor of 18.4%, for all data in the 20-1.24 Å resolution range (Table 1). 91.1 % of residues are within the most favoured region of the Ramachandran plot, no residues are in the disallowed region. The two crystallographically distinct molecules (A and B) are very similar (rms deviation for C~ atoms 0.6 Å). Significant differences are in loops at crystal contact points (Figure 20) (the sidechain conformation of residue Val21 also differs between the A and B molecules, but this is unlikely to affect biological function). Both molecules contain two histamines, bound at sites denoted H and L. The error in the co-ordinates for the majority of nonhydrogen atoms is less than 0.2 Å (Luzzatti, 1952).

The *A* and *B* molecules in the crystal have one extensive contact, main chain hydrogen bonds link a short strand following the α3-helix of molecule *A* (residues *A*143 to *A*146) to the end of strand B of molecule *B* (residues *B*54 to *B*57) (Figure 20a,c). Other hydrogen bonds link *A*150 ND2 to *B*25 0 and *A*167 OG to *B*59 OD 1. These molecules are not related by a two-fold axis. Several residues on the surface of the protein possess multiple conformations in the crystal, however these do not have a role in the structural and functional interactions discussed below. In line with the excellent diffraction, the crystallographic B-factors are rather low (Table 1), indicating that the molecule has a core of considerable rigidity. The refined model for molecule *A* consists of residues *A*1 - *A*171 of the native protein along with the engineered carboxyl-terminal Ile-(His)6 tag. Although the course of the polypeptide chain could be traced in this region, the positions of three of the His side chains was unclear and so they were modelled as Ala. No density was observed for the (His)6 tag in molecule *B* (residue *B*1 was also omitted due to disorder).

Overall Structure

The bulk of the molecule (approximate dimensions 33 Å x 38 Å x 45 Å) is an eight stranded anti-parallel β-barrel, whose topology places FS-HBP2 within the lipocalin superfamily (Figure 13 & Flower, 1996). Lipocalins are, typically, extracellular proteins which transport small hydrophobic ligands with varying degrees of specificity. The HBP β-barrel houses two histamine molecules and the protein is completed by partly helical amino-terminal and carboxyterminal extensions to the β-barrel (Figure 13a). From the high level of sequence similarity, we expect the overall structures of all three Ra-HBPs to be very similar (RMS deviation between Cα atoms ~1 Å).

Unique Features

Several features of functional significance set FS-HBP2 apart from other lipocalins.

The unusually extended amino terminus of FS-HBP2 initially runs along the barrel, forming hydrogen bond interactions with β-strands F and G before forming the α1 and α2 helix. The substantial α2 helix is positioned over the 'mouth' of the barrel, interacting with residues on the rim to form a significant barrier between the binding pockets and the exterior (Figure 20a,b). One side of the helix is held in place by hydrogen bonds to the loop joining strands B and C. The other side of the helix hydrogen bonds to strands F and G (the F-G loop is particularly prominent in HBPs, in line with a functional role).

As with other lipocalins there is an α3-helix, which abuts the mid-point of the barrel. In FS-HBP2, the helix is pinned to the barrel at its extremities by two disulphide bridges (Cys119 to Cysl48 and Cys48 to Cysl68).

Three structurally conserved regions (SCRs), lying in close proximity in the structure, have been identified as determinants of the lipocalin fold (Flower, 1996). SCRI includes a 3/10 helix followed by a Gly-X-Trp sequence in strand A. In FS-HBP2 an α-helix replaces the 3/10 helix and Asn-Val-Tyr (residues 27-29) replaces the signature sequence. In several lipocalins SCR2 contains a Thr-Asp-Tyr motif. Whilst FS-HBP2 does not have this signature sequence there are conformational similarities in this region (such as a characteristic kink in strand F). The final conserved region, between strand H and α3, contains an Arg or Lys residue, nearby the conserved Trp residue of SCRI. In HBP2 this loop contains the tripeptide Thr-Asp-Tyr (139-141), which is usually present in SCR2. Surprisingly, HBP2 contains several other elements of sequence that are also

reminiscent of misplaced lipocalin fragments (although the Glu-Lys-Val-Thr-Ala sequence in strand D is a perfect match with the equivalent portion of epididymal retinoic acid binding protein; Newcomer, 1993).

Perhaps the most striking unique feature of the HBP structure is the presence of the two ligand binding sites within the barrel core. One of the histamine molecules binds across the width of the binding pocket, requiring strands G and H to be stripped away from the rest of the sheet, distorting the canonical hydrogen bonding pattern.

Two Binding Sites which Differ in Affinity for Histamine

To dissect out the relative affinities of the two histamine binding sites, and probe the conformational implications of histamine binding, we attempted to remove histamine from preformed crystals. A rigorous regime of immersion of crystals in mother liquor (minus histamine) was followed by data collection at 100K. The diffraction data revealed that histamine was lost from one of the binding sites in both crystallographically independent molecules whilst the second site remained fully occupied. We infer from this that, in the crystal, one site has a far slower off-rate than the other. The similar behaviour of two molecules in different environments within the crystal strongly suggests that one site, termed H, has a higher intrinsic affinity than the other, termed L. The ligand-protein interactions observed support this assignment.

The binding sites are located, off-centre, at opposing ends of the barrel (Figure 20a). Unusually for a lipocalin (but in accord with the hydrophilic nature of the ligand) both binding pockets sequester a number of charged residues in the interior of the barrel. These residues are critical components of the histamine binding Sites (Figure 21a,b). The RMS difference in the length of the stabilizing hydrogen bonds between the A and B molecules is 0.08 Å; we therefore make no distinction between the two molecules in this respect. The side-chain of residue Tyr100 and a bridging water molecule form a wall separating the H and L sites. Nevertheless a hydrogen bond network links the two sites, the components of which are almost completely conserved between different HBPs. In the H site the histamine ligand lies perpendicular to the long axis of the barrel, leading to distortions in the structure of HBP, compared to other lipocalins. The aromatic side chains of Trp42 and Phe108 are arranged, parallel but slightly off-centre, to form strong Π-Π stacking interactions (McGaughey *et al.*, 1998) with the imidazole of the histamine molecule. The phenolic ring of Tyr100 is perpendicular to the plane of

the imidazole ring of the histamine, contributing further Π-Π interactions. There is an extensive network of hydrogen bonds between the nitrogen atoms of the histamine and the carboxylates of Glu (82 and 135) and Asp (39 and 110) residues, both directly and via bridging water molecules. From its environment, it is expected that the H-site histamine is bound in the di-cationic form.

In the L site the histamine molecule is parallel to the barrel with the imidazole moiety pointing toward the barrel centre. The types of interactions at the L site are similar to the H site but rather less favourable. The imidazole ring of the histamine has base stacking interactions with the phenolic ring of the Phe98 side chain, which are almost perpendicular. There are further van der Waals interactions with the phenolic ring of Tyr29 while Ser20, Asp24 OEl, Tyr100 OH and Aspl2O OE2 form hydrogen bonds with the histamine nitrogen atoms.

Tyr100 therefore has a role in binding both of the histamine molecules, albeit using different modes of interaction. No ordered water molecules are present in the L site and volume calculations suggest that the histamine is slightly less tightly packed in this site. B-factor analysis confirms, however that both histamine ligands are rigidly bound (Table I). The poorer fit of histamine in the L site, raises the question whether a compound other than histamine could be the natural ligand for this pocket.

Structure of FS-HBP2 with One Histamine Bound

The structure of FS-HBP2 without the histamine in the L site was determined in both molecule A and B, although in molecule B a remnant of histamine (approximately 10% occupancy) is seen. The model for this 'semi-apo' structure has an R-factor of 18.7% (Table 1). The structural details are essentially the same for molecule A and B. Four water molecules have replaced the histamine in the L site, one of which takes the position of the histamine Cβ atom (Figure 21c), whilst the others cushion destabilization by occupying the equivalent positions to the nitrogen atoms of the histamine. Removal of the histamine from the L site causes only slight of (of the order of a few tenths of an Angstrom; the overall RMS change in Cot positions is only 0.1 Å) and side chain conformations remain essentially unchanged, (Figure 21c). The histamine remains bound at full occupancy at the H site, consistent with a much higher affinity.

Implications of Sequence Variation within the HBP Family

Sequence variation amongst the three HBPs occurs even for residues directly involved in histamine binding, especially those contributing to the L pocket. Overall FS-HBP2 resembles FS-HBP1 more closely than MS-HBP1, however The L-pocket of FS-HBP2 is in fact more similar to that of MS-HBP1 than FS-HBP1, despite the fact that, overall, FS-HBP2 resembles FS-HBP1 most strongly. In FS-HBP1, histidine and tyrosine residues replace Ser20 and Asp120, respectively. These changes must substantially modify both the shape and charge characteristics of the binding pocket. In contrast to the numerous changes in the L site, only the substitution of Phe 108 for a Leu impinges upon the H site of FS-HBP1. The Kds for FS-HBP2 and MS-HBP1 are similar, and about 10 times lower than that for FS-HBP1, suggesting that modifications at the L site modulate the observed binding affinities. Modelling the FS-HBPI structure indicates that the loss of aromatic residues may open up an internal cavity, presumably changing the specificity.

MS-HBP3, the male specific protein, has more substantial changes compared to FS-HBP2, including insertions and deletions in the loop regions. MS-HBP1 contains an additional Cys (150), which is likely to be on the surface of the molecule and may be responsible for the formation of the covalent dimers discussed above. The putative glycosylation site, Asn61 is also likely to be exposed to the solvent (Figure 13).

Example 5: Design of a synthetic cyclic peptide with histamine binding activity

There are two chains in the histamine binding proteins identified in Figures 17 and 18, A and B. Each chain binds two histamines, one in μmol and one in pmol quantities. Geometrical investigation of the four binding sites is summarised in Tables 2 and 3 below.

These data indicate that in the pmol binding pockets the positively charged quaternary nitrogen tail of histamine is bound to two negatively charged residues (Asp 110 and Glu 135). Similarly, the positively charged imidazole ring is bound to two negatively charged residues (Glu 82 and Asp 39), through the two nitrogens of the ring. There are also two aromatic ring to positive charge interactions between the imidazole ring nitrogen and Trp 42 and Phe 108.

1) Peptide Design

The analysis of the active sites suggested that in order to mimic the histamine binding

sites two separate types of interaction need be considered. The first type of interaction is that between the positively charged centres (nitrogen tail and imidazole ring) and negatively charged residues. The second interaction type being between the positively charged imidazole ring and an aromatic ring.

This led to the design of several cyclic peptide systems incorporating negatively charged residues, e.g. Glu, and aromatic residues e.g. Phe. Initial modelling studies indicated that cyclic hexapeptides would not be sufficiently flexible to allow for histamine recognition. Modelling of cyclic octapeptide systems indicates that they will potentially allow for histamine binding.

The sequence for the suggested cyclic octapeptide is:

Ala-Glu-Ala-Phe-Ala-Glu-Ala-Trp

Figures 17 and 18 show a minimised conformation of the cyclic octapeptide / histamine system. These figures reveal the suggested mode of binding between the histamine and the cyclic peptide. At either 'end' of the cyclic peptide are two negatively-charged Glu residues to interact with the positively charged nitrogen tail and the imidazole ring. The Phe and Trp residues at opposing sides of the imidazole ring allow for aromatic–positive charge interaction.

The interaction distances between the different centres (positive charge to negative charge and positive charge to aromatic group) are shown in Table 2.

Table 2. Average Hst – Peptide Distances

Interaction	Distance Å
Hst Tail -- Glu Acid C	3.66
Imidazole ring – Glu Acid C	3.53
Imidazole ring – Phe Centroid	4.91
Imidazole ring – Trp Centroid	4.94

Atom Numbering System.

Table 3. Strong Binding Sites

Hst Atom	Residue	Atom	A-Chain Å	B-Chain Å
1	Tyr 36	OH	3.32	3.4
1	Asp 110	Acid C	3.67	3.66
1	Glu 135	"	3.88	3.84
2	Glu 82	"	3.33	3.37
3	Asp 39	"	3.44	3.35
3	Trp 42	Centroid of Ring	3.7	3.97
3	Phe 108	"	4.1	4.21

Table 4. Hst Weak Binding Sites

Hst Atom	Residue	Atom	A-Chain Å	B-Chain Å
1	Ser 20	C=O	2.85	2.83
1	Ser 20	OH	3.07	3.13
1	Asp 24	Acid C	2.88	3.12
1	Tyr 29	Centroid	3.23	3.21
1	Asp 120	O	3.64	2.71
2	Asp 24	O	2.64	2.66
3	Tyr 100	OH	2.80	2.78

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CLAIMS

1. A histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which has a binding site comprising amino acid residues phenylalanine, isoleucine or leucine at position I, tryptophan at position II and aspartate or glutamate at positions III and IV wherein residues I to IV are positioned substantially the same as residues 108, 42, 39 and 82 respectively in either of SEQ. ID. Nos 1 or 2, or residues 107, 41, 38 and 78 in SEQ. ID. 3 or residues 122, 54, 50 and 95 in SEQ. ID. 4, and functional equivalents thereof.

2. A histamine or serotonin binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which has a binding site comprising amino acid residues phenylalanine or isoleucine at residue I, tryptophan at residue II and aspartate or glutamate at residues III and IV wherein residues I to IV are positioned substantially the same as residues 98, 137, 24 and 120 respectively in either of SEQ. ID. Nos 1 or 2, or residues 95, 138, 23 and 120 in SEQ. ID. 3 or residues 112, 149, 35 and 135 in SEQ. ID. 4, and functional equivalents thereof.

3. A histamine binding compound capable of binding to histamine or serotonin with a dissociation constant of less than 10^{-7}M and which has two binding sites, the first binding site comprising amino acid residues phenylalanine, isoleucine or leucine at position I, tryptophan at position II and aspartate or glutamate at positions III and IV wherein residues I to IV are positioned substantially the same as residues 108, 42, 39 and 82 respectively in either of SEQ. ID. Nos 1 or 2, or residues 107, 41, 38 and 78 in SEQ. ID. 3 or residues 122, 54, 50 and 95 in SEQ. ID. 4, and the second binding site comprising amino acid residues phenylalanine or isoleucine at residue I, tryptophan at residue II and aspartate or glutamate at residues III and IV wherein residues I to IV are positioned substantially the same as residues 98, 137, 24 and 120 respectively in either of SEQ. ID. Nos 1 or 2, or residues 95, 138, 23 and 120 in SEQ. ID. 3 or residues 112, 149, 35 and 135 in SEQ. ID. 4, and functional equivalents thereof.

4. A histamine binding or serotonin compound according to claim 1 or 3 additionally comprising at residue V, a tyrosine residue, wherein residue V is positioned substantially the same as residue 100 in the sequence of either of SEQ. ID. Nos 1 or 2, residue 97 in SEQ ID 3 or residue 114 in SEQ ID 4, and functional equivalents thereof.

5. A histamine or serotonin binding compound according to claim 2 or 3 additionally comprising at residue V, a tyrosine residue, wherein residue V is positioned substantially the same as residue 29 in the protein sequence of either of SEQ. ID. Nos 1 or 2, residue 28 in SEQ ID 3 or residue 40 in SEQ ID 4, and functional equivalents thereof.

6. A histamine or serotonin binding compound according to any preceding claim wherein said compound is stabilised by either or both of the disulphide bridges formed between cysteines 48 and 169 and cysteines 148 and 119 in the protein sequence of either of SEQ. ID. Nos 1 or 2, cysteines 47 and 175 and cysteines 151 and 119 of SEQ ID 3 or cysteines 162 and 134 of SEQ ID 4.

7. A histamine or serotonin binding compound of any one of the preceding claims which comprises a peptide, or a fragment of any one of the proteins FS-HBP1, FS-HBP2, MS-HBP1 or D.RET6.

8. The histamine or serotonin binding compound of claim 7 that comprises a cyclic peptide.

9. The histamine or serotonin binding compound of claim 8 wherein said cyclic peptide comprises the sequence Ala-Glu-Ala-Phe-Ala-Glu-Ala-Trp.

10. The histamine or serotonin binding compound of any one of claims 1 to 9 that comprises a synthetic compound.

11. A protein comprising the Ra-Res amino acid sequence given in SEQ. ID. No. 5 or functionally equivalent derivative or functionally equivalent fragment thereof.

12. A protein comprising the Av-HBP amino acid sequence given in SEQ. ID. No. 6 or functionally equivalent derivative or functionally equivalent fragment thereof.

13. A protein comprising the Ih/Bm-HBP1 amino acid sequence given in SEQ. ID. No. 7 or functional equivalent derivative or fragment thereof.

14. A protein comprising the Ih/Bm-HBP2 amino acid sequence given in SEQ. ID. No. 8 or functional equivalent derivative or fragment thereof.

15. A protein comprising the Ih/Bm-HBP3 amino acid sequence given in SEQ. ID. No. 9 or functional equivalent derivative or fragment thereof.

16. A protein comprising the Ih/Bm-HBP4 amino acid sequence given in SEQ. ID. No. 10 or functional equivalent derivative or fragment thereof.

17. A protein comprising the Ih/Bm-HBP5 amino acid sequence given in SEQ. ID. No. 11 or functional equivalent derivative or fragment thereof.

18. The histamine or serotonin binding compound of any one of claims 1 to 10 or protein according to any one of claims 11 to 17 produced by recombinant DNA technology.

19. A histamine or serotonin binding compound or protein according to any one of the preceding claims that binds specifically to histamine.

20. The histamine or serotonin binding compound or protein of any one of the preceding claims having an effector or reporter molecule attached thereto.

21. The histamine or serotonin binding compound or protein of any preceding claim that is derived from blood-feeding ectoparasites, spiders, scorpions or snakes and venomous animals.

22. The histamine or serotonin binding compound or protein of claim 21 that is derived from ticks.

23. The histamine or serotonin binding compound or protein of claim 22 that is derived from Ixodid ticks.

24. The histamine or serotonin binding compound or protein of claim 23 that is derived from *Rhipicephalus appendiculatus, D. reticulatus, Amblyomma variegatum, Boophilus microplus or Ixodes hexagonus.*

25. The histamine or serotonin binding compound or protein of any one of the preceding claims associated with one or more carbohydrate moieties.

26. The histamine or serotonin binding compound or protein of any one of the preceding claims that is associated with one or more peptides or polypeptides.

27. The histamine or serotonin binding compound or protein of claim 26 that is genetically or chemically fused to one or more peptides or polypeptides.

28. The histamine or serotonin binding compound or protein of any one of the preceding claims attached to a label or toxin.

29. The histamine or serotonin binding compound or protein of any one of the preceding claims that is bound to a support, such as a resin.

30. A therapeutic or diagnostic composition comprising a histamine or serotonin binding compound or protein according to any one of the preceding claims.

31. A therapeutic or diagnostic composition according to claim 30 additionally comprising serotonin.

32. A therapeutic or diagnostic composition according to claim 31 additionally comprising a cysteinyl leukotriene, platelet activating factor, or a thromboxane.

33. A vaccine comprising a histamine or serotonin binding compound according to any one of claims 1-10 or protein according to any one of claims 11-17.

34. The histamine or serotonin binding compound or protein according to any one of claims 1 to 29 or composition of any one of claims 30 to 32 for use in therapy.

35. The histamine or serotonin binding compound or protein according to any one of claims 1 to 29 for use as a pharmaceutical.

36. Use of the histamine or serotonin binding compound or protein according to any one of claims 1 to 29 as a pharmaceutical.

37. The histamine or serotonin binding compound or protein according to any one of claims 1 to 29 for use in a vaccine.

38. Use of the histamine or serotonin binding compound or protein according to any one of claims 1 to 29 in a vaccine.

39. The histamine or serotonin binding compound or protein of any one of claims 1 to 29 for use in the detection or quantification of histamine in human, animal, plant, and food material

40. The histamine or serotonin binding compound of any one of claims 1 to 29 for use in the depletion or removal of histamine from food products, cell cultures or human, animal, plant and food material.

41. The histamine or serotonin binding compound of any one of claims 1 to 29 for use in the binding or detection of histamine in humans or animals.

42. The histamine or serotonin binding compound or protein of any one of claims 1 to 29 for use as an anti-histamine agent, an anti-inflammatory drug or in the treatment of allergy.

43. The histamine or serotonin binding compound or protein of any one of claims 1 to 29 for use as a tool in scientific research concerning the role of histamine in biological processes .

44. The use of a histamine or serotonin binding compound according to any one of claims 1 to 29 in conjunction with a pharmaceutically-acceptable carrier in the manufacture of a medicament for the treatment or prevention of inflammation or allergic reaction in humans or animals.

45. A nucleic acid compound which encodes a histamine or serotonin binding molecule or protein according to any one of claims 1 to 29 or which hybridises with said nucleic acid molecule under standard hybridisation conditions.

46. The nucleic acid molecule of claim 45 which comprises DNA, cDNA or RNA.

47. A cloning or expression vector comprising a nucleic acid molecule according to either of claims 45 or 46.

48. The vector of claim 47 which is virus based.

49. A host cell transformed or transfected with the vector of either of claims 47 or 48.

50. A transgenic animal that has been transformed by a nucleic acid molecule according to either of claims 45 or 46 or vector according to either of claims 47 or 48.

FIG. 1

FS-HBP1

```
T3→
   1 AGAAAGCCAACATGAAGCTTCTGCTCTCTCTTGCCTTCGTCTTAGCTCTCAGCCAAGTTA   60
                M  K  L  L  L  S  L  A  F  V  L  A  L  S  Q  V  K

  61 AAGCCGATAAGCCAGTTTGGGCGGATGAAGCGGCAAACGGGGAACACCAAGACGCCTGGA  120
     A  D  K  P  V  W  A  D  E  A  A  N  G  E  H  Q  D  A  W  K
        ↑

 121 AGCATCTCCAAAAACTCGTTGAAGAGAATTACGACTTGATAAAAGCCACCTACAAGAACG  180
     H  L  Q  K  L  V  E  E  N  Y  D  L  I  K  A  T  Y  K  N  D

                                          T3a→←T7c
 181 ACCCAGTTTGGGGTAACGACTTCACTTGCGTGGGTACTGCAGCGCAGAATTTGAACGAGG  240
     P  V  W  G  N  D  F  T  C  V  G  T  A  A  Q  N  L  N  E  D

 241 ACGAGAAGAACGTTGAAGCATGGTTTATGTTTATGAATAATGCTGATACCGTATACCAAC  300
     E  K  N  V  E  A  W  F  M  F  M  N  N  A  D  T  V  Y  Q  H

 301 ATACTTTTGAAAAGGCGACTCCTGATAAAATGTACGGTTACAATAAGGAAAACGCCATCA  360
     T  F  E  K  A  T  P  D  K  M  Y  G  Y  N  K  E  N  A  I  T

 361 CATATCAAACAGAGGATGGGCAAGTTCTCACAGACGTCCTTGCATTCTCTGACGACAATT  420
     Y  Q  T  E  D  G  Q  V  L  T  D  V  L  A  F  S  D  D  N  C

 421 GCTATGTCATCTACGCTCTTGGCCCAGATGGAAGTGGAGCAGGTTACGAACTCTGGGCTA  480
     Y  V  I  Y  A  L  G  P  D  G  S  G  A  G  Y  E  L  W  A  T

                                T3b→←T7d
 481 CCGATTACACGGATGTTCCAGCCAGTTGTCTAGAGAAGTTCAATGAGTATGCTGCAGGTC  540
     D  Y  T  D  V  P  A  S  C  L  E  K  F  N  E  Y  A  A  G  L

 541 TGCCGGTACGGGACGTATACACAAGTGATTGCCTCCCAGAATAACTTGGGCATATCGTAA  600
     P  V  R  D  V  Y  T  S  D  C  L  P  E  *

 601 TTTCAACTTCAAAGTGTGTTATTGTCAGCATATGTCTCGAGTGTTTGATGTAGTGCGTTC  660

 661 GATGATGCCATTCATCTAGGTTTCGGGTGTTCGGTACTTTATGGTCACTGCCGACGGCCA  720

                                                          ←T7
 721 GCACGAGTACTCGAAAATAAAGTATTCTGAAATCGGAAAAAAAAAAAAAAA 770
```

FIG. 2

FS-HBP2

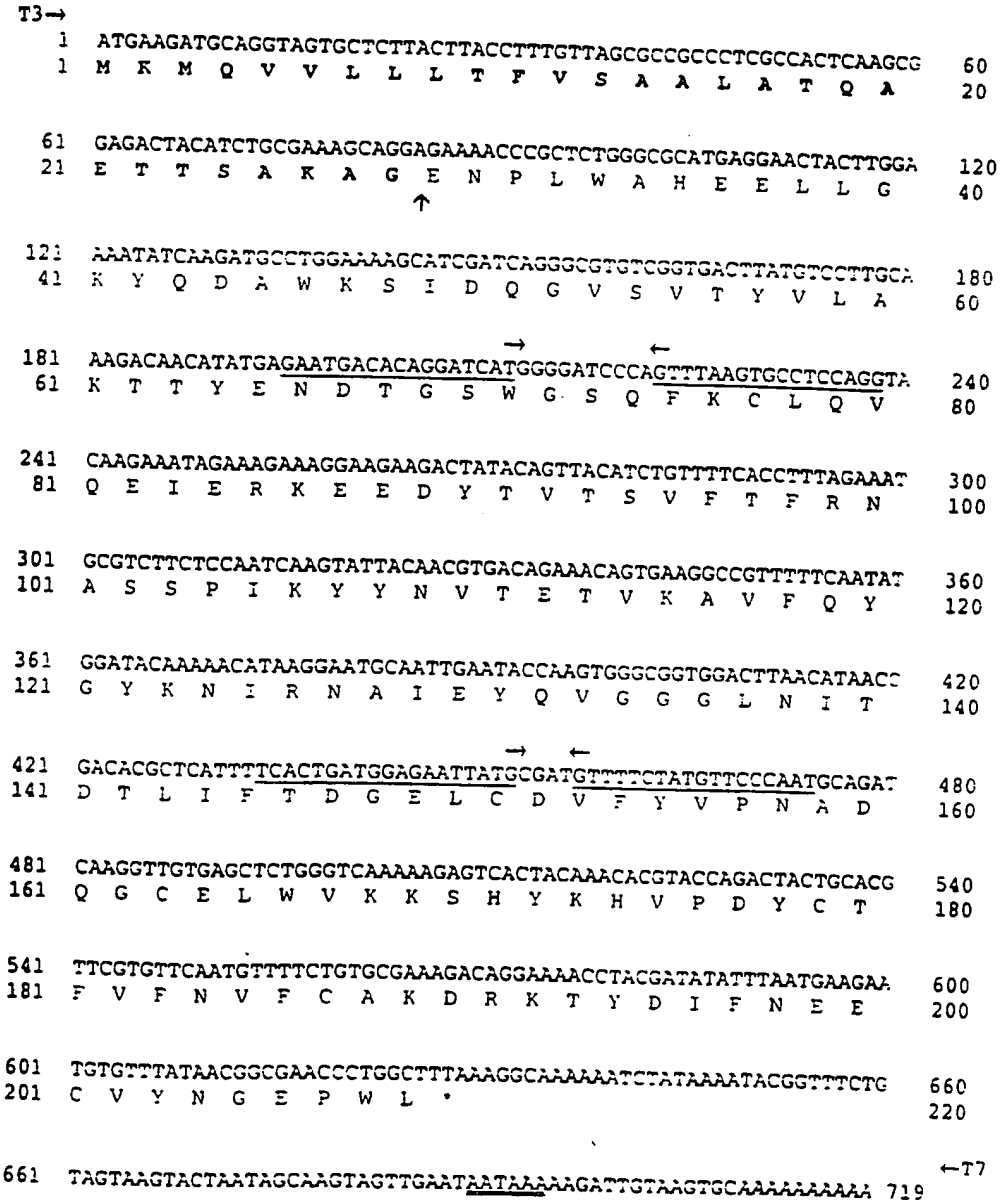
```
T3→
   1 GCCGCGACGGAACTTCGAAGGAAGTCAGCATGAAGCTTCTCATACTCTCTCTTGCCCTCG  60
                                M  K  L  L  I  L  S  L  A  L  V

  61 TCCTCGCCCTCAGCCAGGTTAAGGGAAATCAGCCAGATTGGGCCGATGAAGCGGCAAATG 120
     L  A  L  S  Q  V  K  G  N  Q  P  D  W  A  D  E  A  A  N  G
                              ↑

 121 GTGCACACCAAGACGCCTGGAAGAGTCTGAAAGCGGACGTTGAAAACGTTTACTACATGG 180
     A  H  Q  D  A  W  K  S  L  K  A  D  V  E  N  V  Y  Y  M  V

 181 TGAAGGCCACCTATAAGAATGACCCAGTGTGGGGCAATGACTTCACTTGCGTGGGTGTTA 240
     K  A  T  Y  K  N  D  P  V  W  G  N  D  F  T  C  V  G  V  M

                T3b→ ←T7a
 241 TGGCAAATGATGTCAACGAGGATGAGAAGAGCATTCAAGCAGAGTTTTTGTTTATGAATA 300
     A  N  D  V  N  E  D  E  K  S  I  Q  A  E  F  L  F  M  N  N

 301 ATGCTGACACAAACATGCAATTCGCCACTGAAAAGGTGACTGCTGTTAAAATGTATGGTT 360
     A  D  T  N  M  Q  F  A  T  E  K  V  T  A  V  K  M  Y  G  Y

 361 ACAATAGGGAAAACGCCTTCAGATACGAGACGGAGGATGGCCAAGTTTTCACAGACGTCA 420
     N  R  E  N  A  F  R  Y  E  T  E  D  G  Q  V  F  T  D  V  I

                                        →
 421 TTGCATACTCTGATGACAACTGCGATGTCATCTACGTTCCTGGCACAGACGGAAATGAGG 480
     A  Y  S  D  D  N  C  D  V  I  Y  V  P  G  T  D  G  N  E  E

                ←
 481 AAGGTTACGAACTATGGACTACGGATTACGACAACATTCCAGCCAATTGTTTAAATAAGT 540
     G  Y  E  L  W  T  T  D  Y  D  N  I  P  A  N  C  L  N  K  F

 541 TTAATGAGTACGCTGTAGGTAGGGAGACAAGGGATGTATTCACAAGTGCTTGCCTAGAGT 600
     N  E  Y  A  V  G  R  E  T  R  D  V  F  T  S  A  C  L  E  *

          →                                   ←
 601 AATAACTTCAGAATGTCGTTCTTTCAAAGCGAAAAACCAACAATGTGAACATCGGCTTGC 660

 661 TGTGCTCGACGTAGCCAGCGATAATGTTGTTTTCCTGGGTTTCTGGGTTTGGATACTTTT 720

 721 AGCCACTGCCGAAGAGCTGTAAAGGTAATGAAAAATAAAATGTTCAAGAGTGTGAAAAAA 780

                ←T7
 781 AAAAAAAAAAAAA 793
```

FIG. 3

MS-HBP1

```
                T3→
1   AAAGCACTCAACATGAAGGTTCTTTTGTTGGTTCTTGGAGCTGCTCTTTGCCAGAATGCA   60
                M  K  V  L  L  L  V  L  G  A  A  L  C  Q  N  A

61  GATGCAAACCCAACATGGGCGAACGAAGCTAAATTGGGATCCTACCAAGACGCCTGGAAG  120
    D  A  N  P  T  W  A  N  E  A  K  L  G  S  Y  Q  D  A  W  K
          ↑

121 AGCCTTCAGCAAGACCAAAACAAGAGATACTATTTGGCACAAGCGACACAAACGACTGAC  180
    S  L  Q  Q  D  Q  N  K  R  Y  Y  L  A  Q  A  T  Q  T  T  D

                                    →
181 GGCGTATGGGGTGAAGAGTTTACTTGTGTGAGTGTTACGGCTGAGAAGATTGGAAAGAAA  240
    G  V  W  G  E  E  F  T  C  V  S  V  T  A  E  K  I  G  K  K

          ←
241 AAACTTAACGCTACGATCCTCTATAAAAATAAGCACCTTACTGACCTGAAAGAGAGTCAT  300
    K  L  N  A  T  I  L  Y  K  N  K  H  L  T  D  L  K  E  S  H
          ═══

301 GAAACAATCACTGTCTGGAAAGCATACGACTACACAACGGAGAATGGCATCAAGTACGAG  360
    E  T  I  T  V  W  K  A  Y  D  Y  T  T  E  N  G  I  K  Y  E

361 ACGCAAGGGACAAGGACGCAGACTTTCGAAGATGTCTTTGTATTCTCTGATTACAAGAAC  420
    T  Q  G  T  R  T  Q  T  F  E  D  V  F  V  F  S  D  Y  K  N

                →                                       ←
421 TGCGATGTAATTTTCGTTCCCAAAGAGAGAGGAAGCGACGAGGGCGACTATGAATTGTGG  480
    C  D  V  I  F  V  P  K  E  R  G  S  D  E  G  D  Y  E  L  W

481 GTTAGTGAAGACAAGATTGACAAGATTCCCGATTGCTGCAAGTTTACGATGGCGTACTTT  540
    V  S  E  D  K  I  D  K  I  P  D  C  C  K  F  T  M  A  Y  F

                →
541 GCCCAACAGCAGGAGAAGACGGTTCGTAATGTATACACTGACTCATCATGCAAACCAGCA  600
    A  Q  Q  Q  E  K  T  V  R  N  V  Y  T  D  S  S  C  K  P  A

601 CCAGCTCAGAACTGATATTCTGGTAATGCTTGAACCGTAATGGTTCGACCTGCAGTCTAG  660
    P  A  Q  N  *

661 AAACATTTACCACCATCACGGTGATTATCTTACCGTAGTTTCTTAGGTCTTGTTCTTTGA  720

                                                ←T7
721 ATAAAATAGTTCCCTGCATTGACAAAAAAAAAA  753
```

FIG. 4


T3→
1 ATGAAGATGCAGGTAGTGCTCTTACTTACCTTTGTTAGCGCCGCCCTCGCCACTCAAGCG 60
1 M K M Q V V L L L T F V S A A L A T Q A 20
61 GAGACTACATCTGCGAAAGCAGGAGAAAACCCGCTCTGGGCGCATGAGGAACTACTTGGA 120
21 E T T S A K A G E N P L W A H E E L L G 40
121 AAATATCAAGATGCCTGGAAAAGCATCGATCAGGGCGTGTCGGTGACTTATGTCCTTGCA 180
41 K Y Q D A W K S I D Q G V S V T Y V L A 60
181 AAGACAACATATGAGAATGACACAGGATCATGGGGATCCCAGTTTAAGTGCCTCCAGGTA 240
61 K T T Y E N D T G S W G S Q F K C L Q V 80
241 CAAGAAATAGAAAGAAAGGAAGAAGACTATACAGTTACATCTGTTTTCACCTTTAGAAAT 300
81 Q E I E R K E E D Y T V T S V F T F R N 100
301 GCGTCTTCTCCAATCAAGTATTACAACGTGACAGAAACAGTGAAGGCCGTTTTTCAATAT 360
101 A S S P I K Y Y N V T E T V K A V F Q Y 120
361 GGATACAAAAACATAAGGAATGCAATTGAATACCAAGTGGGCGGTGGACTTAACATAACC 420
121 G Y K N I R N A I E Y Q V G G G L N I T 140
421 GACACGCTCATTTTCACTGATGGAGAATTATGCGATGTTTTCTATGTTCCCAATGCAGAT 480
141 D T L I F T D G E L C D V F Y V P N A D 160
481 CAAGGTTGTGAGCTCTGGGTCAAAAAGAGTCACTACAAACACGTACCAGACTACTGCACG 540
161 Q G C E L W V K K S H Y K H V P D Y C T 180
541 TTCGTGTTCAATGTTTTCTGTGCGAAAGACAGGAAAACCTACGATATATTTAATGAAGAA 600
181 F V F N V F C A K D R K T Y D I F N E E 200
601 TGTGTTTATAACGGCGAACCCTGGCTTTAAAGGCAAAAAATCTATAAAATACGGTTTCTG 660
201 C V Y N G E P W L • 220
661 TAGTAAGTACTAATAGCAAGTAGTTGAATAATAAAAAGATTGTAAGTGCAAAAAAAAAA 719
←T7

Ra-Res

FIG. 5

```
1   CAACTGATCACTAAAATGTTCCTTGCGGGTTTCTTCATTTTCGGCGCTGCCGTCCTCTCA   60
                M  F  L  A  G  F  F  I  F  G  A  A  V  L  S

61  GTTTTGGCTGAGGAGACACCTAATGATAGATGTACTACACACACTCCTAATGGATGGCAG   120
    V  L  A  E  E  T  P  N  D  R  C  T  T  H  T  P  N  G  W  Q

121 TTTCTCAAGAAAGGCAAGAGATACGATATGAAACAGAGAACCTTCCAAACACCTAACTCA   180
    F  L  K  K  G  K  R  Y  D  M  K  Q  R  T  F  Q  T  P  N  S

181 GACGACACTAAATGCCTGTCCAGTACTATCGACGGAAAGAATGAAAATAACCATACAGTA   240
    D  D  T  K  C  L  S  S  T  I  D  G  K  N  E  N  N  H  T  V,

241 CAAGCAACGATAAGATATCGAAATGGTTATGAAGGAAAATGGGACACCATCCGCCAGGAG   300
    Q  A  T  I  R  Y  R  N  G  Y  E  G  K  W  D  T  I  R  Q  E

301 TACGAGTTCCCCAACTACACTGCAGGAGACTACAACTCCATGAAGACAACAGACAAATCC   360
    Y  E  F  P  N  Y  T  A  G  D  Y  N  S  M  K  T  T  D  K  S

361 CCGCCTCCGCCGGCATCATACCTGTTTGGATATACTGGAAGCTCTTGTGCCGTGGTGTAC   420
    P  P  P  P  A  S  Y  L  F  G  Y  T  G  S  S  C  A  V  V  Y

421 GTGAATTCCATTGGACCTGTTCGTAGCAATTCTGAAAACCCACCAGAAAGACTCACAGCA   480
    V  N  S  I  G  P  V  R  S  N  S  E  N  P  P  E  R  L  T  A

481 AGTCAGGAAAGTGCACAACGCGATTGCGTCCTTTGGGTCGATCACGATGAAAAAGCTACC   540
    S  Q  E  S  A  Q  R  D  C  V  L  W  V  D  H  D  E  K  A  T

541 CAAGAACAATGCTGTGAAGATTTCTTCAAGACCCACTGCAAAGAGACTGTCCATGTCATA   600
    Q  E  Q  C  C  E  D  F  F  K  T  H  C  K  E  T  V  H  V  I

601 TACGACGTGAATAGATGCAAGGAGAATGGCAGTGAATAACACGATGCCGGGAATGGCATG   660
    Y  D  V  N  R  C  K  E  N  G  S  E  *

661 GCGACTTCATTTATGAAGGAAGACTTCCACAGATGTGAAACTTGCCTTCATTTTGCTTGT   720

721 TACTTTAGACCAACATATTCTTCCTTTTCCGACTTCAATGATATGATCTAGGTTGTAAAA   780

781 AGAGCGTTTTAATAAAGAAAGTATTAGCATCGATGATGGAAATATAAAAAAAA 832
```

Av-HBP

FIG. 6

```
1   GCGACCGCGCCCAGCCGTACAGAACAAATAGCCTTCGTTGCAAACGTGCAGCGTAGTCGG   60

61  ATGCCTAGTTAAACACCACACACACGTAAAAAGTAGACGAAACTGGCTTCGCTTCCAGCA   120

121 CCAAGCAGGTCATCGTCTGGTCCACTGACGATGAACTCTGCCTTGTGGGTTTTACTAGGA   180
                                  M  N  S  A  L  W  V  L  L  G

181 TCATCCTTATGGCTGCATACGGTAGCGTTCATGATTCCCACATGGGCAGATGAAGGCAGG   240
    S  S  L  W  L  H  T  V  A  F  M  I  P  T  W  A  D  E  G  R

241 TTTGGCAAGTACCAGAACGCCTGGAAGGCCCTGAATCAGCGGATTAACACAACACATGTC   300
    F  G  K  Y  Q  N  A  W  K  A  L  N  Q  R  I  N  T  T  H  V

301 CTTGTGAGGTCAACGTATATCGACAATCCATATTTATGGGGCAAGAACTTCTCATGCGTA   360
    L  V  R  S  T  Y  I  D  N  P  Y  L  W  G  K  N  F  S  C  V

361 CGCGCTCGAACTGTCGAAGTCTTTCCCAGCAGCAAGACTGTGGAACTGGAGTTTAGTTTC   420
    R  A  R  T  V  E  V  F  P  S  S  K  T  V  E  L  E  F  S  F

421 AGAAACAGGACTGGTATATTGTGCATGAGAAATCAAACGGTTCGAGCTGGAAAGGATTAC   480
    R  N  R  T  G  I  L  C  M  R  N  Q  T  V  R  A  G  K  D  Y

481 TTTTATCATCAGCCTAACGCCTTCGAATTCATGCTGAGAGGTAACAGGTCGTTTTCTAAC   540
    F  Y  H  Q  P  N  A  F  E  F  M  L  R  G  N  R  S  F  S  N

541 GCTGTCATGTTTACCGACGGAATGACATGTAATCTGCTCAGCTTTCCATACCAGCGCAAC   600
    A  V  M  F  T  D  G  M  T  C  N  L  L  S  F  P  Y  Q  R  N

601 AAACCACAATGCGAACTATGGGTGAAGGACACGCGCGTCGACAACATTCCCCCTTGTTGC   660
    K  P  Q  C  E  L  W  V  K  D  T  R  V  D  N  I  P  P  C  C

661 TCGTTCATGTTCGACTATTTGTGCCCACAGCCTCGTCCATTCATCATTTACGACAAAGCA   720
    S  F  M  F  D  Y  L  C  P  Q  P  R  P  F  I  I  Y  D  K  A
```

```
721  ATGTGCACGGTGAGGCCACCCCGCTAGAAAGAAAAGGGATGAAAAGGCTACTCGAAGAAG  780
     M  C  T  V  R  P  P  R  *

781  CAACAACCAATCAGTGCCCACAAGAGAACCGTTCCAGTCCTGCGAAAGTTGCGCCTCCCA  840

841  AAACACATACATTTCACTGCAAAGATGACCGATGCAGTCGCAAATTCGTGTCCTAGAACT  900

901  CAAGTGCTGTTTTGGAAACTCGGAAAGGAGACAGTAGAAGCTAACTGCTGTGATACCTAG  960

961  GCCAGGCATTTCCGTCGGGCACTGTTTTTTATGAATAGGGTAGGGTGAAAGTATTTTGGC  1020

1021 TTTGCTGTGGCCCAATAAATAGCGTATATTAGCGGACTAGCATCGAAGTTCCAGATGCTA  1080

1081 TAAAGCAGCTAAAACTCACTTCTGCCTGGAACTTCGATAGGTATTGAATAGATCATGCGC  1140

1141 GCACAGAAAAGAAAAGTATCAATCAAAACATAAAAAGCATTCTTCGCATGTGCGCAAAGC  1200

1201 ATTCCCTAAGTCCACGCTAAAAATAGGTGTCATTTCATATAGCATCGAGTTCTATACGTT  1260

1261 CTTAAGATGCTACCGGTCATTCATTCCTTTCTCGTCTATGCCTCATGGATCTGAACCAAG  1320

1321 TTCTTCTATTGCCTCCTTGTTTTCCGGTAGCTACAGAGTTCAGCAGCACCATTGCTAGTG  1380

1381 CATATTTTATCTTCGTGCTGTGTTTGTCGCAGTATATTTTTCTGCCTATTCACGATATTT  1440

1441 GCACAATGTAATAAAACATTTGCCTGCCTAAAAAAAAAAAAAAAAAAAA  1488
```

FIG. 6(contd.)

Ih/Bm-HBP1

FIG. 7

```
1   CTCCAGCTCTGCTTCGACGATGAAGGCTCTCCTGATCGCTGTCGGCTACCTGGCTGCCGT   60
                      M  K  A  L  L  I  A  V  G  Y  L  A  A  V

61  CACAGCGGCACCCCAAGCTTCGCCTTCCTCTCCGAGGAACGAACCACTCAAGAATACTAC  120
    T  A  A  P  Q  A  S  P  S  S  P  R  N  E  P  L  K  N  T  T

121 GTGGCACAGCAAGGAACTGAAAAATTATCAAGATGCGTGGAAGTCCATCAATCAAAACGT  180
    W  H  S  K  E  L  K  N  Y  Q  D  A  W  K  S  I  N  Q  N  V

181 CAGCACTACCTACTACTTCCTCAGATCAACCTACAACAACGACAGTGTCTGGGGTAAAAA  240
    S  T  T  Y  Y  F  L  R  S  T  Y  N  N  D  S  V  W  G  K  N

241 TTTCACCTGTCTTAGCGTCACGGTGACATCGAAACATGAATCAACGTTCACCGTCGAATA  300
    F  T  C  L  S  V  T  V  T  S  K  H  E  S  T  F  T  V  E  Y

301 TAACACCACGTACAAAAATCAGAGCCAACAATGGGTCAGCATGACGGAAAACGTCACGGC  360
    N  T  T  Y  K  N  Q  S  Q  Q  W  V  S  M  T  E  N  V  T  A

361 CGTGCAGGAGGAGGGCTACGACGTTAAAAATATCATTCAGTGGACAACAGAGAATAACAC  420
    V  Q  E  E  G  Y  D  V  K  N  I  I  Q  W  T  T  E  N  N  T

421 AAAGTTCAATGATACTGTTGTTTTTACGGACGGCCAGACTTGTGATCTGTTGTACATCCC  480
    K  F  N  D  T  V  V  F  T  D  G  Q  T  C  D  L  L  Y  I  P

481 GTACAAAGAAAACGGTTACGAGCTGTGGGTGCGTTCGGATTACCTGCAGAACACTCCAAC  540
    Y  K  E  N  G  Y  E  L  W  V  R  S  D  Y  L  Q  N  T  P  T

541 GTGCTGCCAGTTCATCTTTGACCTCGTCGCATTGGGACGTACCACGTACAATATCTCCAC  600
    C  C  Q  F  I  F  D  L  V  A  L  G  R  T  T  Y  N  I  S  T

601 TCCTGACTGCGTGACCAAAACCTCTCGTTAGACCGTGAAAGCCGCGGCTTATGCTACTCG  660
    P  D  C  V  T  K  T  S  R  *

661 ACTGCTCAGGTTGGAAGAGTAGGGAGCCCCGACGCGCACTACTACTAAAAATGATTCCAA  720

721 ATAAAGTATTCAAACATTTCAAAAAAAAAAAAAAAAAAAAA                     760
```

Ih/Bm-HBP2

FIG. 8

```
1   AGTGACTCCTGCTCTGCTTCGACGATGAAGGCTCTCCTGATCGCTGTCGTCTACCTGACT   60
                          M  K  A  L  L  I  A  V  V  Y  L  T

61  GCCGTCACAGCGGCAGACCAAGCTCCGCCTTCCTCTACGAGGAATGAACCACTCGAGAAA  120
    A  V  T  A  A  D  Q  A  P  P  S  S  T  R  N  E  P  L  E  K

121 ACTACCTGGCACAACCAGACACTGGGACGTTATCAAGATGCGTGGAAGTCCATCAATCAA  180
    T  T  W  H  N  Q  T  L  G  R  Y  Q  D  A  W  K  S  I  N  Q

181 AGCGTCGGCACTACCTACTACTTCCTCAGATCAACCTACAACAACGACAGCGTGTGGGGT  240
    S  V  G  T  T  Y  Y  F  L  R  S  T  Y  N  N  D  S  V  W  G

241 AAAAATTTCACCTGTCTTAGCGTCACGGTGACATCGAAATATGAATCAACGTTCACCGTC  300
    K  N  F  T  C  L  S  V  T  V  T  S  K  Y  E  S  T  F  T  V

301 GAATATAACACCACGTACAAAAATCAGAGCCAACAATGGGTCAGCATGTCGGAAAACGTC  360
    E  Y  N  T  T  Y  K  N  Q  S  Q  Q  W  V  S  M  S  E  N  V

361 ACGGCCGTGCAGGAGGGCGGCTACAGTGTTAAAAACATCATTCAGTGGACAACGGAGAAT  420
    T  A  V  Q  E  G  G  Y  S  V  K  N  I  I  Q  W  T  T  E  N

421 AACACAAAGTTCAATGATACTGTTGTTTTTACGGACGGCCAGACTTGTGATGTGTTATAC  480
    N  T  K  F  N  D  T  V  V  F  T  D  G  Q  T  C  D  V  L  Y

481 ATCCCGTACAAAGAAGACGGTTACGAGCTGTGGGTGCGTTCGGAATACCTGCAGAACACT  540
    I  P  Y  K  E  D  G  Y  E  L  W  V  R  S  E  Y  L  Q  N  T

541 CCAACGTGCTGCCAGTTCATCTTTGACCTCGTCGCATTGGGACGTACCACGTACAATATC  600
    P  T  C  C  Q  F  I  F  D  L  V  A  L  G  R  T  T  Y  N  I

601 TCCACTCCTAACTGCGTGGCCACCACCGCTGGTTAGACAATGCAAGCCGCGGCTTAATTT  660
    S  T  P  N  C  V  A  T  T  A  G  *

661 ACTCGACCGCTCAGGTTGGAAGTGCCGGGAGCCTCGACGGGCACTACTACTTAAAATGAT  720

721 TTCGAATAAAGTATTCAAGCATTTCTGGAAAAAAAAAAAAAAAAA  765
```

FIG. 9

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```
1   GATGGCGCTCAGATTTGCACTTCTGCTGGCGTGCATCGTCACGGCATGTGGCTGGAGAAC   60
      M  A  L  R  F  A  L  L  L  A  C  I  V  T  A  C  G  W  R  T

61  ACGGATTCAAGAGAAAGGTCCCGAGAACAACCCTCTCATGAACACCCAACGTTTGGGAAA  120
      R  I  Q  E  K  G  P  E  N  N  P  L  M  N  T  Q  R  L  G  K

121 AATGCAAGACGCATGGAAGAGTCTGGAAAAGGCAACAAATCAGTCGTATGTCTTGGTGTT  180
      M  Q  D  A  W  K  S  L  E  K  A  T  N  Q  S  Y  V  L  V  F

181 CCGCTCAAGAAATCACGAACCAGAGATATCCTGCGTGTACGTGAGGGCTAGTAATATAAA  240
      R  S  R  N  H  E  P  E  I  S  C  V  Y  V  R  A  S  N  I  N

241 TAATGACACTAAAACTGCAACTTATACCAGAACATATTACAATATGACGGCAAACGCAAC  300
      N  D  T  K  T  A  T  Y  T  R  T  Y  Y  N  M  T  A  N  A  T

301 CATGACGGTGAATTATACTGCAAGAGCTCTGAAGCAAGTGGACTATGAGTCGGAAAATGT  360
      M  T  V  N  Y  T  A  R  A  L  K  Q  V  D  Y  E  S  E  N  V

361 CGTACGAGTAAACCTGACAGGTGGGGTCCCCAGCAACGATACAGTTCCTCTTGGAAGCTA  420
      V  R  V  N  L  T  G  G  V  P  S  N  D  T  V  P  L  G  S  Y

421 CGAATACGTCGAGTACGGTAATTACTCCTGCAATAGCTCATCGACACCCTTTTTGGATGC  480
      E  Y  V  E  Y  G  N  Y  S  C  N  S  S  S  T  P  F  L  D  A

481 TGTGCAAATGGCATCGCAAGGGCAATCCAGAGGGCCGGATATCGAAGGGCGCACATATCT  540
      V  Q  M  A  S  Q  G  Q  S  R  G  P  D  I  E  G  R  T  Y  L
```

FIG. 9 (CONTD.)

```
541  AGACTTCTACGTCGTCTACAATCAACCATCGTGCAATGTCCTGAAGTCCCCGCTCCTGGG  600
       D  F  Y  V  V  Y  N  Q  P  S  C  N  V  L  K  S  P  L  L  G

601  AGGTGCTTGTGACTTTTGGGTGACAGAATCCGAGTTGCAAAAAGCACTAAATAAGACATC  660
       G  A  C  D  F  W  V  T  E  S  E  L  Q  K  A  L  N  K  T  S

661  AGAGAAGAAAAAAACAAAGCTAGAAGCGAGAGCAAGGAAAGCTGGAGGAGATTCCGATGA  720
       E  K  K  K  T  K  L  E  A  R  A  R  K  A  G  G  D  S  D  D

721  CCAGGGACCTGAACTGGAGGTCGTCTTCAAAAATCTGCCCCCTCCCTGCCGCGCAGCGTT  780
       Q  G  P  E  L  E  V  V  F  K  N  L  P  P  P  C  R  A  A  F

781  CATAACTTCCTGCGGCTATCCAACTTTTCTTATGTACAACAAGACCATCTGTAATCGAAC  840
       I  T  S  C  G  Y  P  T  F  L  M  Y  N  K  T  I  C  N  R  T

841  GGATTCTGCTGCGGTGTGAACGTCCCCTGCGAGCAAGTAGAACGTCCGTGAAGACAGCAG  900
       D  S  A  A  V  *

901  GAAGATAGTTGACTGTTTTGTTGGCGGAATGTGACTACTAGTCTGAATCATTAAAAAGAT  960

961  TCNGCTGACGGGTGTGGCGGGAACTTTTTTAAATGAAATTGGTCATACTTGTTGAAAGAC  1020

1021 AAAAATAAAACAATATGTTACTCCTC  1046
```

Ih/Bm-HBP4

FIG. 10

```
1   GGAAACCAGGATGGCGCTCAGATTTGCACTTCTGCTGGCGTGCATCGTCACGGCATGTGG   60
              M  A  L  R  F  A  L  L  L  A  C  I  V  T  A  C  G

61  CTGGAGAACACGGATTCAAGAGAAAGGTCCCGAGAACAACCCTCTCATGAACACCCAACG  120
     W  R  T  R  I  Q  E  K  G  P  E  N  N  P  L  M  N  T  Q  R

121 TTTGGGAAAAATGCAAGACGCATGGAAGAGTCTGGAAAAGGCAGCAAATCAGACGTATGT  180
     L  G  K  M  Q  D  A  W  K  S  L  E  K  A  A  N  Q  T  Y  V

181 CTTGGTGTTCCGCTCAAGAAATCACGAACCAGATATATCCTGCGTCTACGTGAGAGCTAG  240
     L  V  F  R  S  R  N  H  E  P  D  I  S  C  V  Y  V  R  A  S

241 TAATTTAGATAATGCAACTAAAACTGCAGATTATACCAGAACATATTACAATATGACGGC  300
     N  L  D  N  A  T  K  T  A  D  Y  T  R  T  Y  Y  N  M  T  A

301 AAAACAAAACGTGTCGGTAAATTATACTGCAAGAGCTCTGAAGCAAGTGGACTATGAGTC  360
     K  Q  N  V  S  V  N  Y  T  A  R  A  L  K  Q  V  D  Y  E  S

361 GGAAAATGTCGTACGAGTAAACCTGACAGGTGGGGTCCCCAGTAACGATACAGTTCCTCC  420
     E  N  V  V  R  V  N  L  T  G  G  V  P  S  N  D  T  V  P  P

421 TGGAAGCTTCGAATACGTCGAGTACGGTAATTACTCCTGCAATAGCTCATCGACACCCTT  480
     G  S  F  E  Y  V  E  Y  G  N  Y  S  C  N  S  S  S  T  P  F

481 TTTGGATGCTGTGCAAATGGCATCGCAAGGGCAATCCTGGGGGCCGGATGTCGAAGGGCG  540
     L  D  A  V  Q  M  A  S  Q  G  Q  S  W  G  P  D  V  E  G  R
```

```
541  CACATATCTAGATTTCTACGTCGTCTACAATCAACCGTCGTGCAATGTCCTGAAGTCCCC  600
       T  Y  L  D  F  Y  V  V  Y  N  Q  P  S  C  N  V  L  K  S  P

601  GCTCCTGGGAGGTGCTTGTGACTTCTGGGTGCCACAATCAGAGTTGGACAAGGTACTAAA  660
       L  L  G  G  A  C  D  F  W  V  P  Q  S  E  L  D  K  V  L  N

661  CAAAAAAGGAGATAAGAAAAAGCCAGCTAAGTCAAGCAGTCAAAATGGAGACGAAGGTTC  720
       K  K  G  D  K  K  K  P  A  K  S  S  S  Q  N  G  D  E  G  S

721  TGATGCCGAGCAACCTGAACTGGAGGCCATCTTTAAACATCTACCCCCTCCCTGCCGCGC  780
       D  A  E  Q  P  E  L  E  A  I  F  K  H  L  P  P  P  C  R  A

781  AGCGTTCATAACTTCCTGCGGCTATCCAAATTTTCTCATGTACAACAAGACGATCTGTAA  840
       A  F  I  T  S  C  G  Y  P  N  F  L  M  Y  N  K  T  I  C  N

841  TGCAGCGGGTCATGCTGCGAACTGAACGTCCTCTGCGAACGAGTAGAGCGTGCGTAAAAA  900
       A  A  G  H  A  A  N  *

901  CAACTGGTCTGAATCTTTTAAGAAATTCGGCAAAGTGCGGGTGGCGCGAACTTTTATCAA  960

961  ACTGGTCATACATGTGAAAGAAAAAAATAAAACAAAATGTGCATAAAAAAAAAAAAAAAA  1020

1021 AAAAA  1025
```

FIG. 10(contd.)

FIG. 11

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```
1   CGAAGAGCAGGTACGATTCGAATCTTTGCAATGGACATTCGCAGCGCTGTTTTGTTCGCG   60
                                  M  D  I  R  S  A  V  L  F  A

61  TGCATCGTCTCGGCGTGTTGTGGCTTTTGGCGCTGGACAACACGGAGGGTAACTAAAAAG  120
    C  I  V  S  A  C  C  G  F  W  R  W  T  T  R  R  V  T  K  K

121 CCTGATAACAGCCCTCTGTTGAACAACCAACATCTTGGTCTTTTCCAGGACGCATGGAAG  180
    P  D  N  S  P  L  L  N  N  Q  H  L  G  L  F  Q  D  A  W  K

181 ACTATAGAAGAGACGTCCAATGATACGTATGTCCTGATGTTCCGCTCAAAACATTACGAC  240
    T  I  E  E  T  S  N  D  T  Y  V  L  M  F  R  S  K  H  Y  D

241 CACGAGAACAAGGCTAAATGTGTCTTCGTAACGGCAAATATTACTGACTCCCGGAACAAA  300
    H  E  N  K  A  K  C  V  F  V  T  A  N  I  T  D  S  R  N  K

301 ACTGCCAATTACACAATAACGTATTACGATACTACAACAAATACATCCAACAATTTTACA  360
    T  A  N  Y  T  I  T  Y  Y  D  T  T  T  N  T  S  N  N  F  T

361 ATCCCAGTGAGAGCTCTGAACCAAACTGACTACTCACTAGAAAATGTGATTCGAGCAAGC  420
    I  P  V  R  A  L  N  Q  T  D  Y  S  L  E  N  V  I  R  A  S

421 TTCAACGGCGACACTCCAAGCTCTACTCCAGCCCCTCCCGGAAGCAGCGTGTACATTCAG  480
    F  N  G  D  T  P  S  S  T  P  A  P  P  G  S  S  V  Y  I  Q

481 TATAATAATGTTACCTGCTACGCCCAATATCACCCATTTTCAAATAATGGAATCAGTGCA  540
    Y  N  N  V  T  C  Y  A  Q  Y  H  P  F  S  N  N  G  I  S  A
```

FIG. 11 (CONTD.)

```
541  AAATATGATGAAATGCCCCGGGATGGCCGAAATTACTTGTTCGACAATTTTATTGGTGCT  600
     K  Y  D  E  M  P  R  D  G  R  N  Y  L  F  D  N  F  I  G  A

601  TACTTGGACTTCTACGTGGTGTTCAGCCAGCCGACATGCAACGTTCTCAGAGTCCGAGAA  660
     Y  L  D  F  Y  V  V  F  S  Q  P  T  C  N  V  L  R  V  R  E

661  GGATGTGACTTCTGGCTAAGGAAAACTGAGTTGCCAAGCCTACTGAAAGCAGCAGAAAAT  720
     G  C  D  F  W  L  R  K  T  E  L  P  S  L  L  K  A  A  E  N

721  GATGACAACGATAACACGGAATCGCTGAAGAACTATTGGGAAAGAAGAATAAATAATACT  780
     D  D  N  D  N  T  E  S  L  K  N  Y  W  E  R  R  I  N  N  T

781  AAAACAAGATTTCGACATAATACTAAGAAATGTAAGATGTACGTACAACGTTATTCAATT  840
     K  T  R  F  R  H  N  T  K  K  C  K  M  Y  V  Q  R  Y  S  I

841  GAGAAGGCTGAAGATGTCTTTAAAAACACTGCTTTTAAACACCTCCCCTCCGACTGCCGC  900
     E  K  A  E  D  V  F  K  N  T  A  F  K  H  L  P  S  D  C  R

901  TTTGCCTTCCTGGCCGCTTGTGGAAATCCAGCATTCACAATATACGACCCAGAAACATGT  960
     F  A  F  L  A  A  C  G  N  P  A  F  T  I  Y  D  P  E  T  C

961  AATAGCTCCCTGCCAGCTAATATGGCAGAAAGTTAAATGAGCTATTTCACTTCATGTTCG 1020
     N  S  S  L  P  A  N  M  A  E  S  *

1021 ACCGTATGCCTGGTATGCAAGAAGGTGAGGTTGGACAGGATACTTCCGAATTATTTTTTC 1080

1081 AGTCTGCCTTGTACGCACGAAATAACAAAATATCTGTTGAAGCCNNCAACNNNNNNAANA 1140

1141 ANAAAAAANAAAAAAA 1156
```

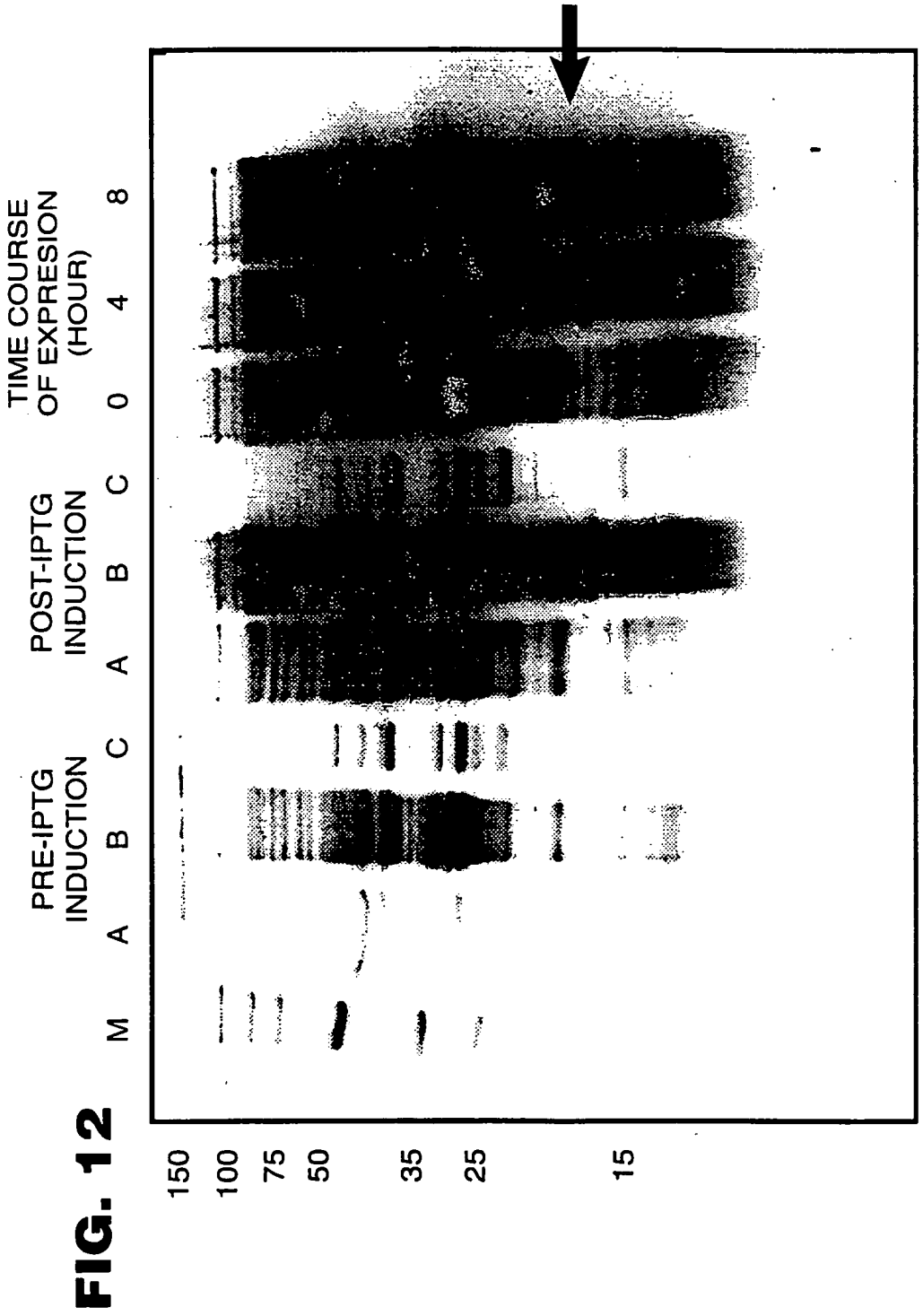
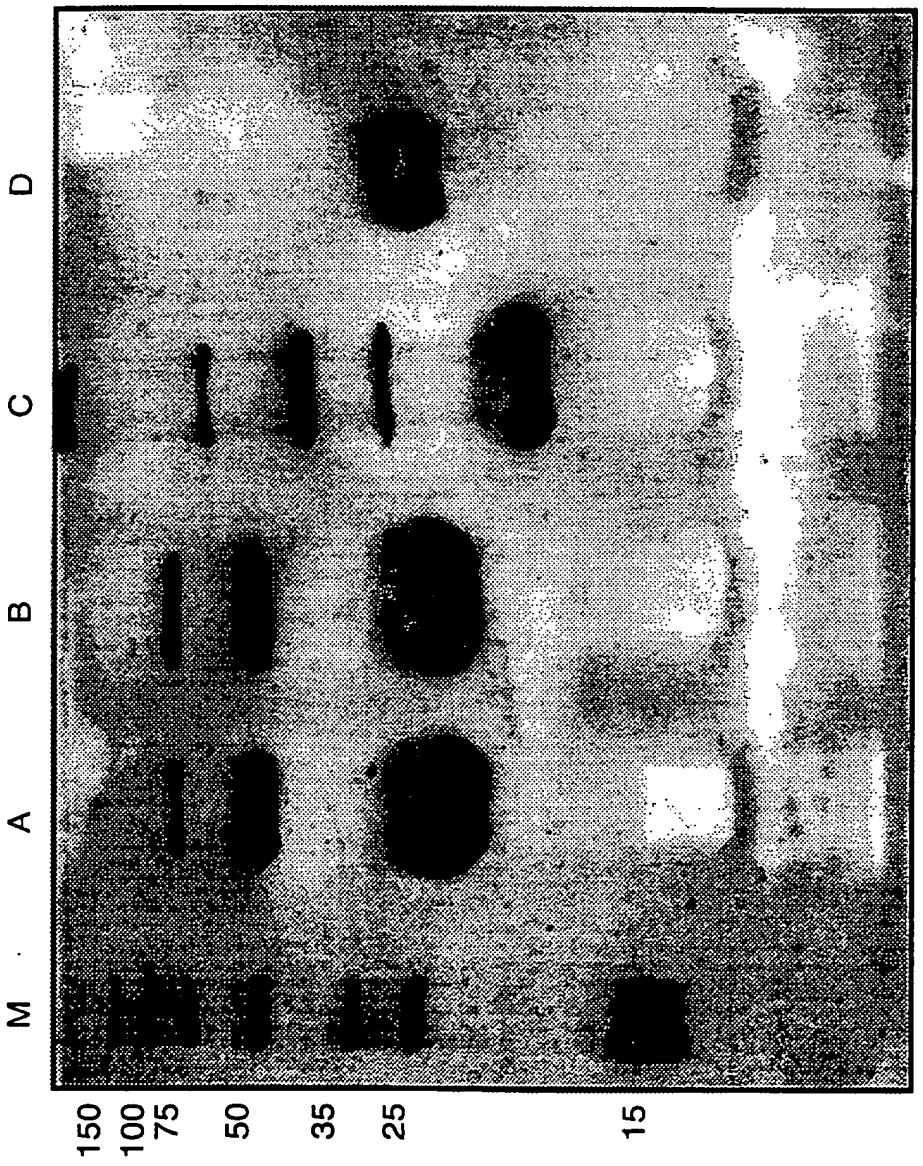

M
PRE-IPTG INDUCTION
A
B
C
POST-IPTG INDUCTION
A
B
C
TIME COURSE OF EXPRESION (HOUR)
0
4
8
150
100
75
50
35
25
15

FIG. 12

FIG. 13



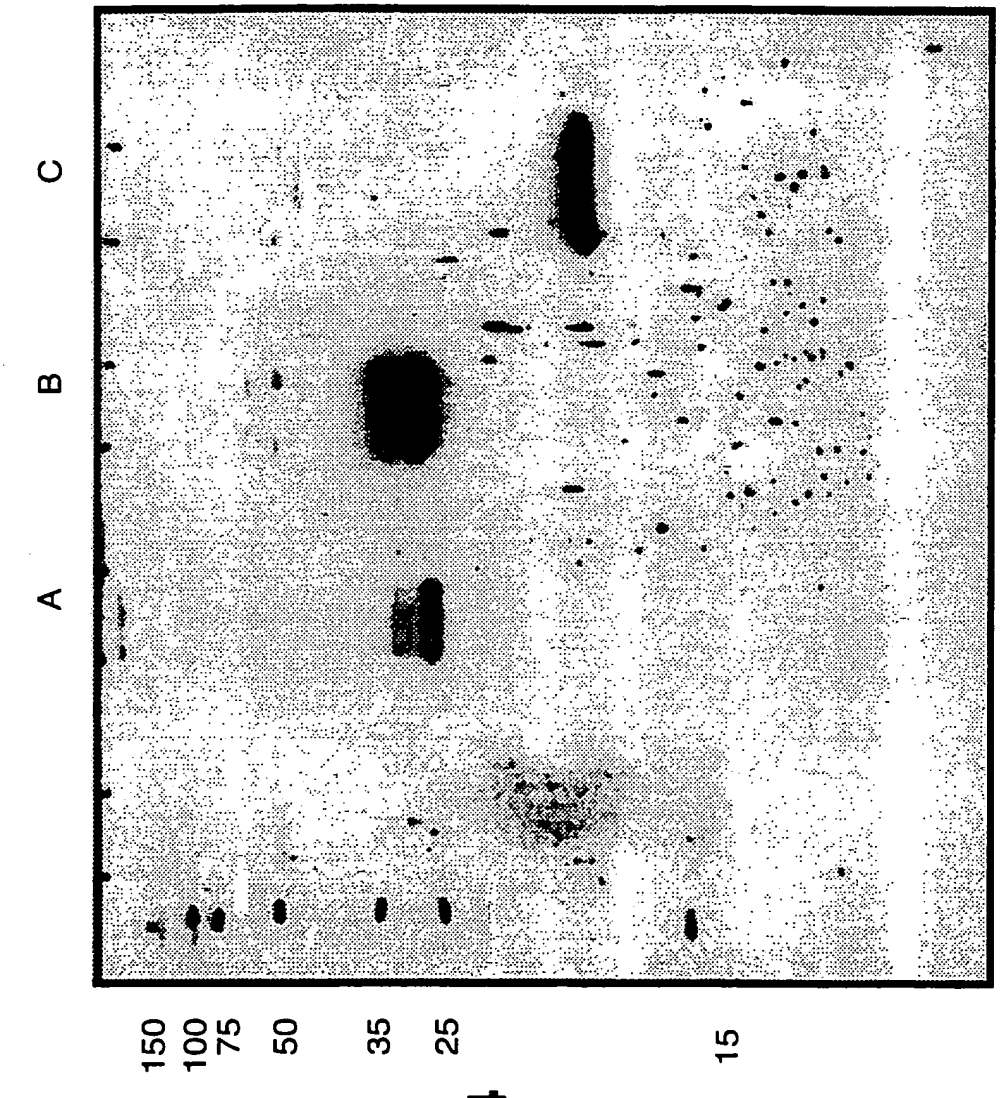


FIG. 14

FIG. 15

Binding Activity of derretine to histamine and its methylsubstitutions

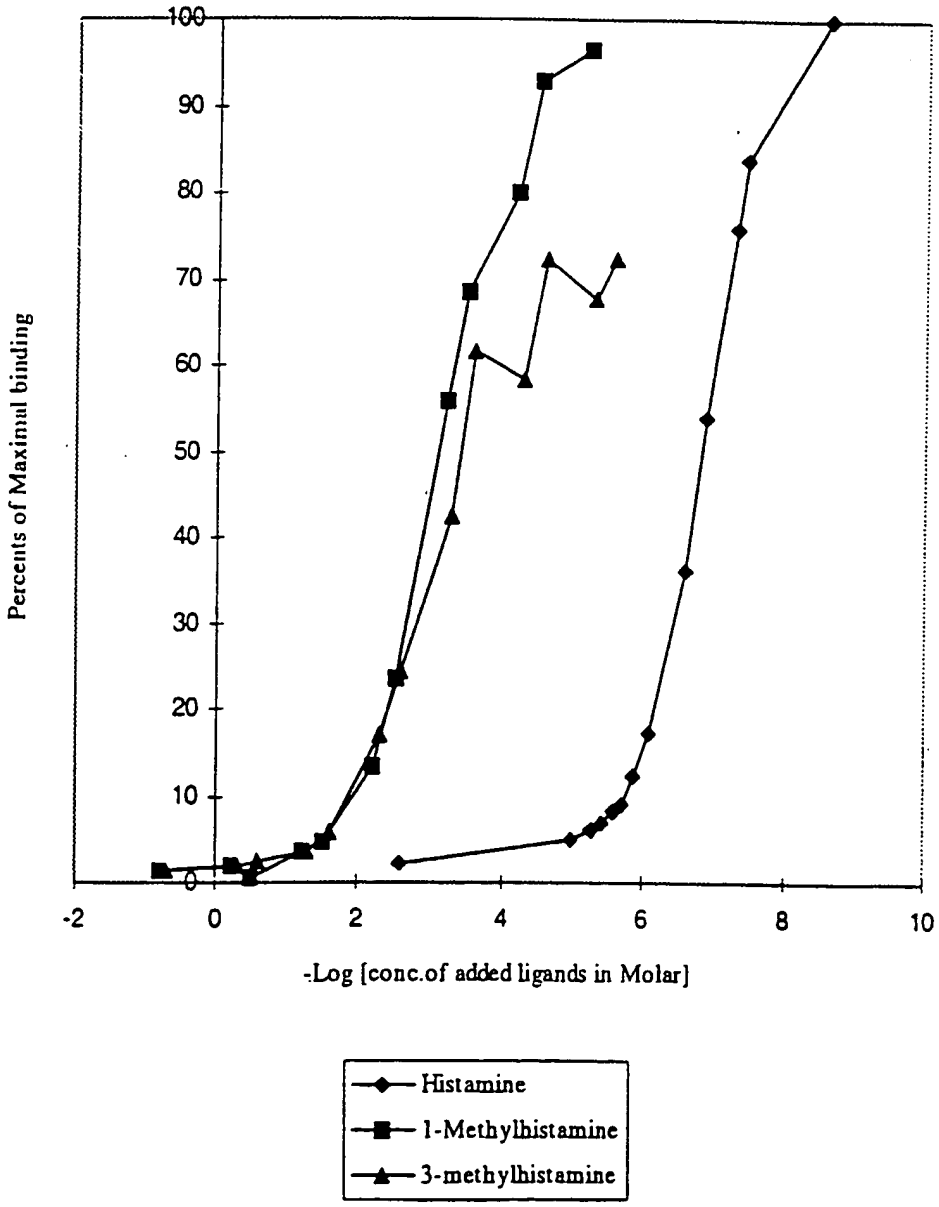
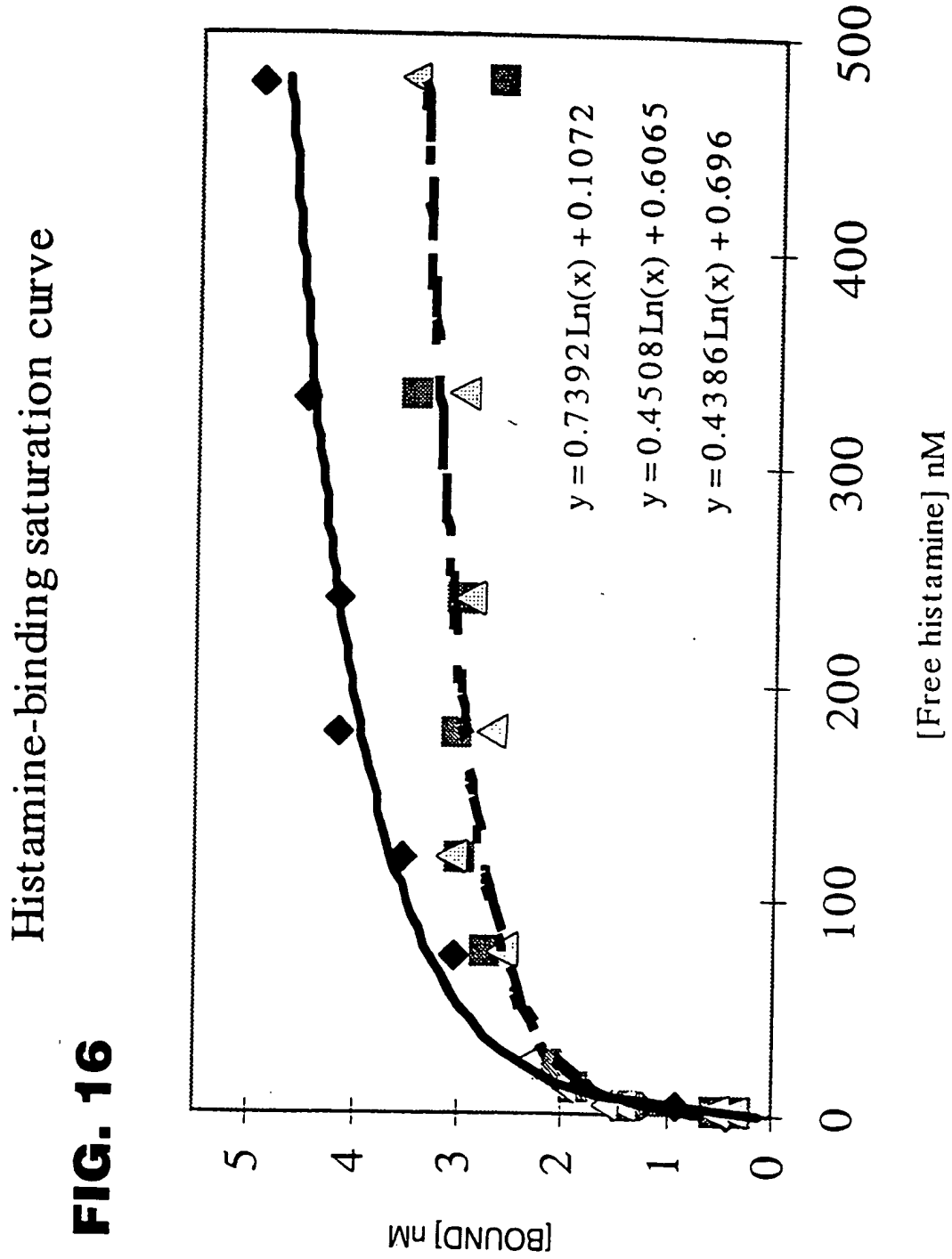


FIG. 16


Histamine-binding saturation curve
[BOUND] nM
0
1
2
3
4
5
0
100
200
300
400
500
[Free histamine] nM
y = 0.7392Ln(x) + 0.1072
y = 0.4508Ln(x) + 0.6065
y = 0.4386Ln(x) + 0.696

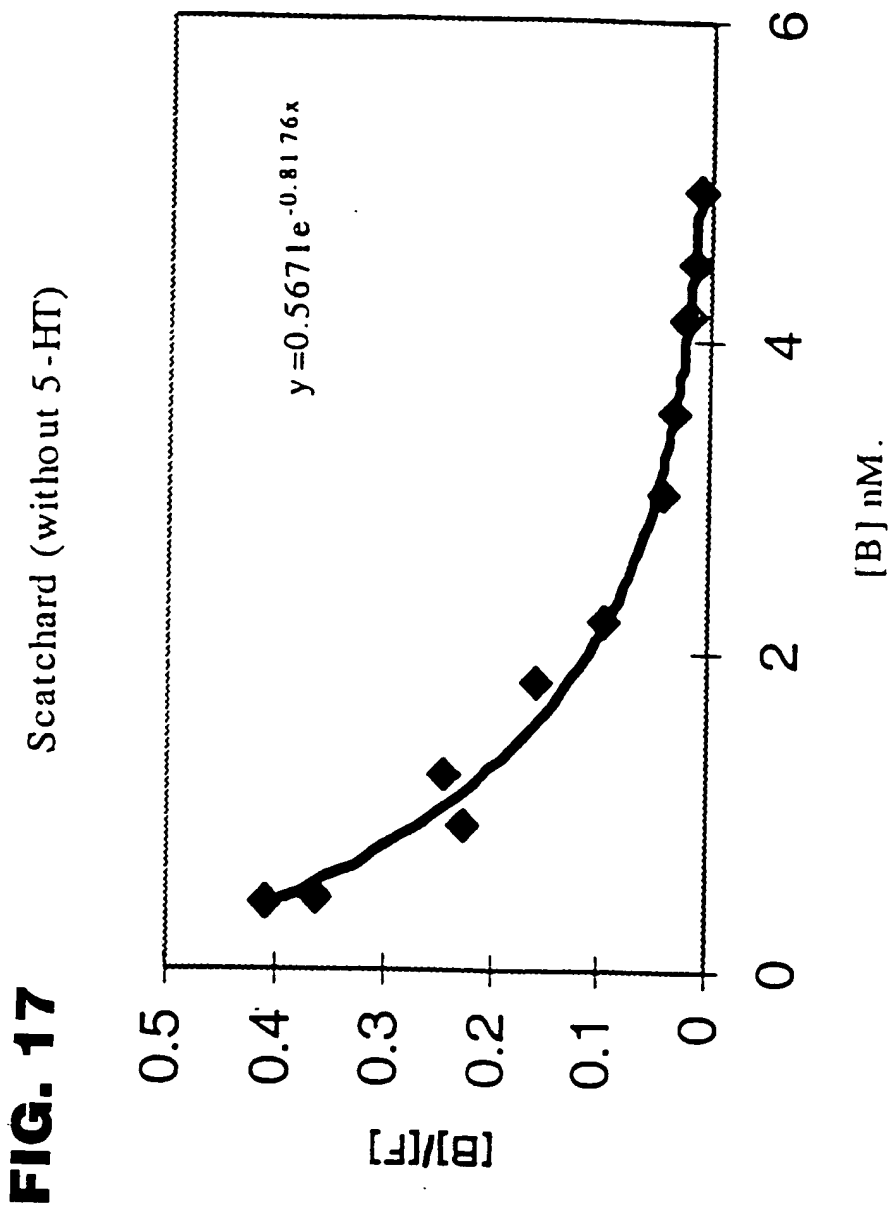
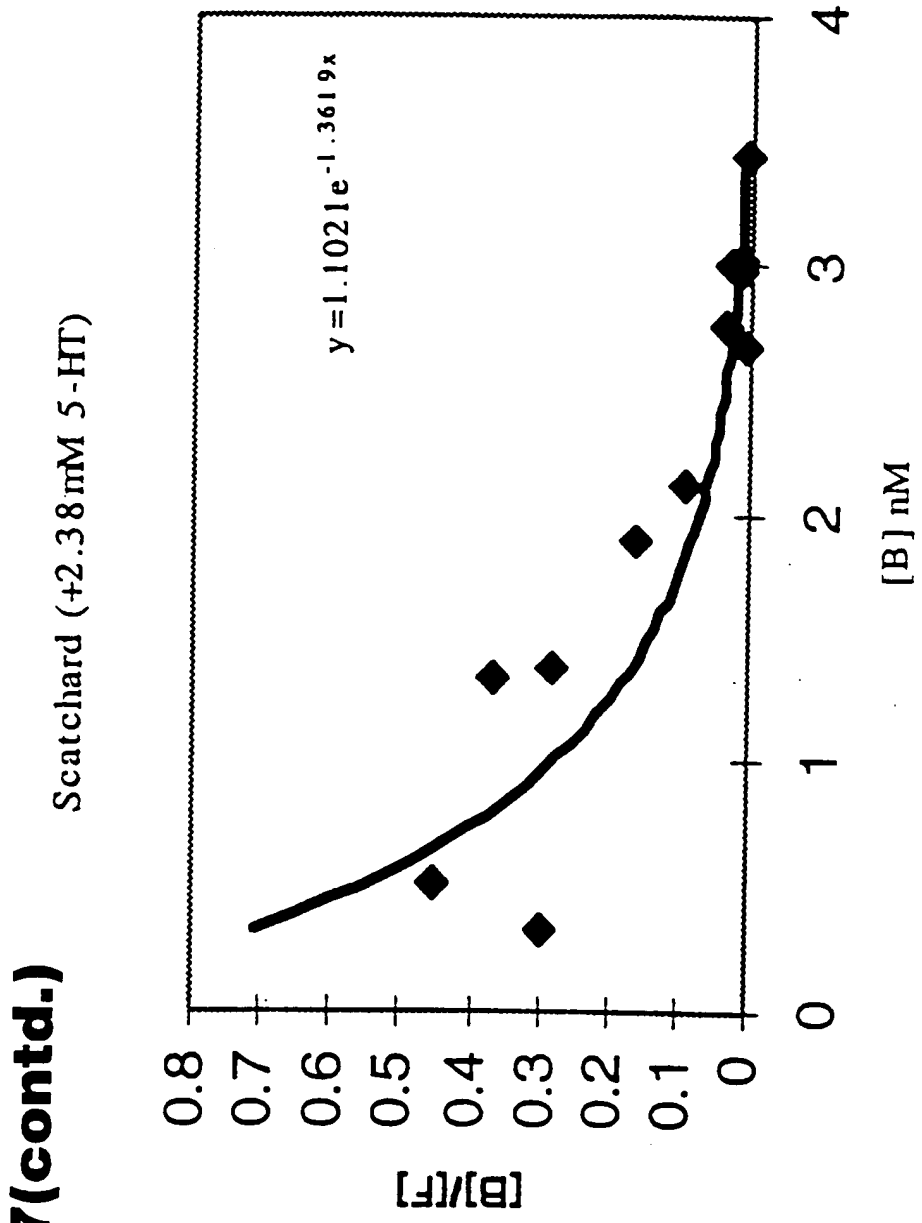

Scatchard (without 5-HT)
y =0.5671e-0.8176x
[B]/[F]
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
[B] nM.

FIG. 17

FIG. 17(contd.)


Scatchard (+2.38mM 5-HT)
y=1.1021e-1.3619x
[B]/[F]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
[B] nM

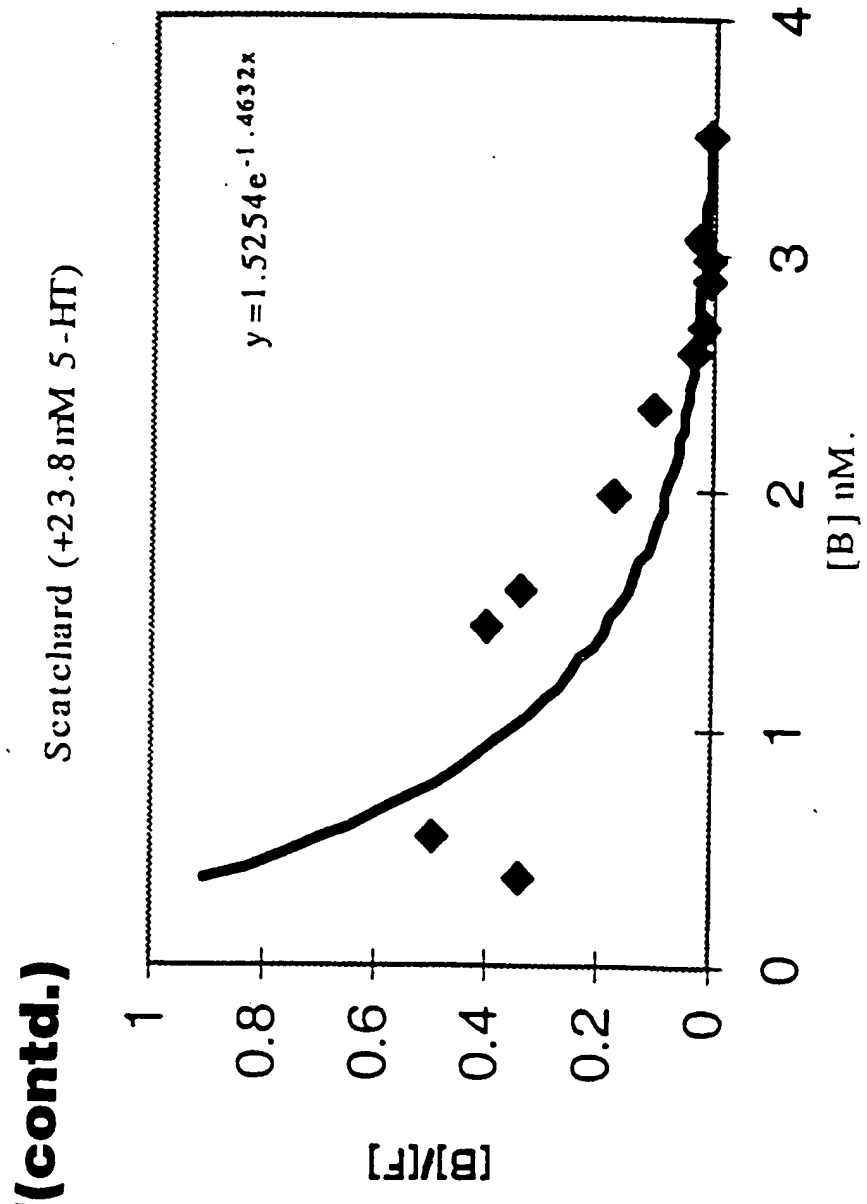

Scatchard (+23.8 nM 5-HT)
y = 1.5254e-1.4632x
[B]/[F]
[B] nM.
0
0.2
0.4
0.6
0.8
1
0
1
2
3
4

FIG. 17(contd.)

FIG. 18

FIG. 19

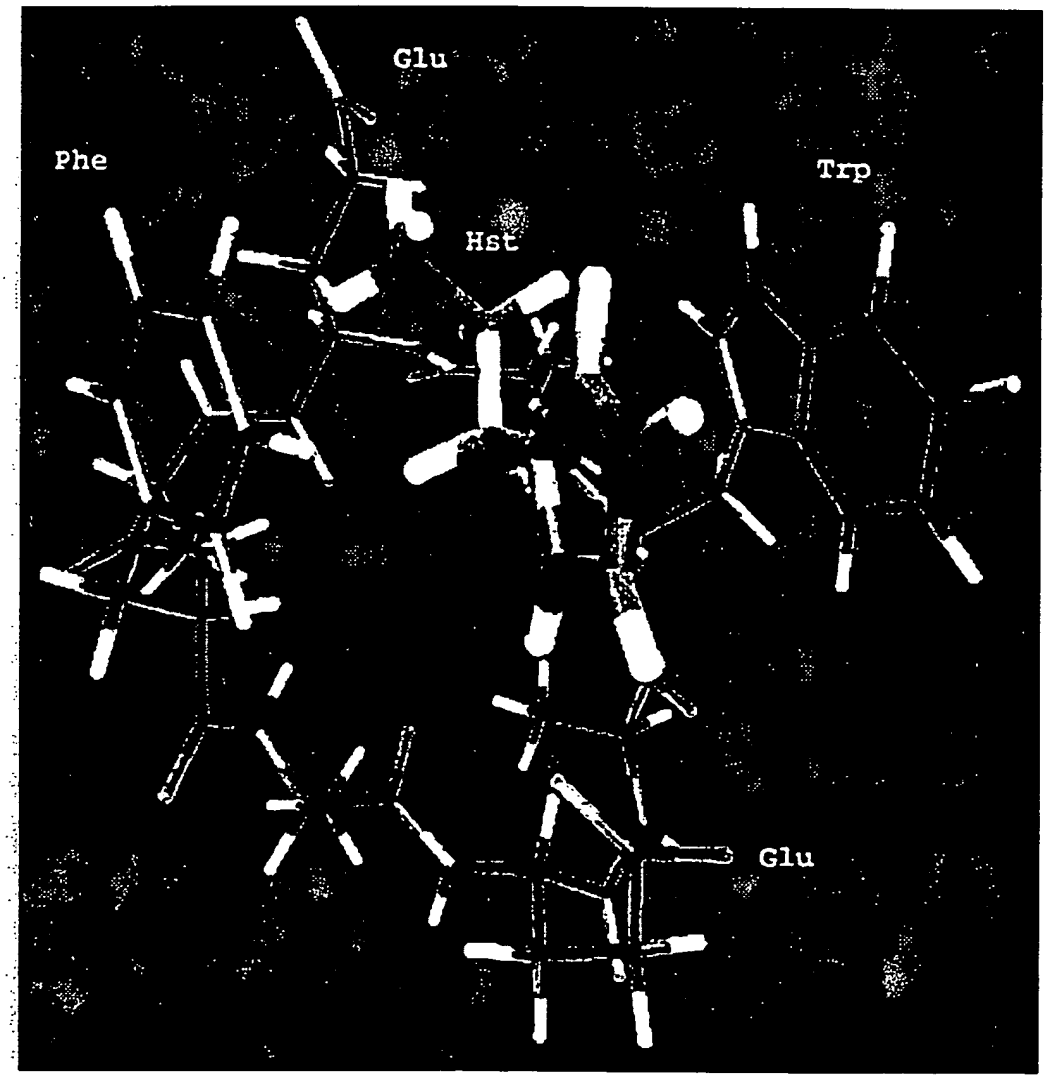


FIG. 20(a)

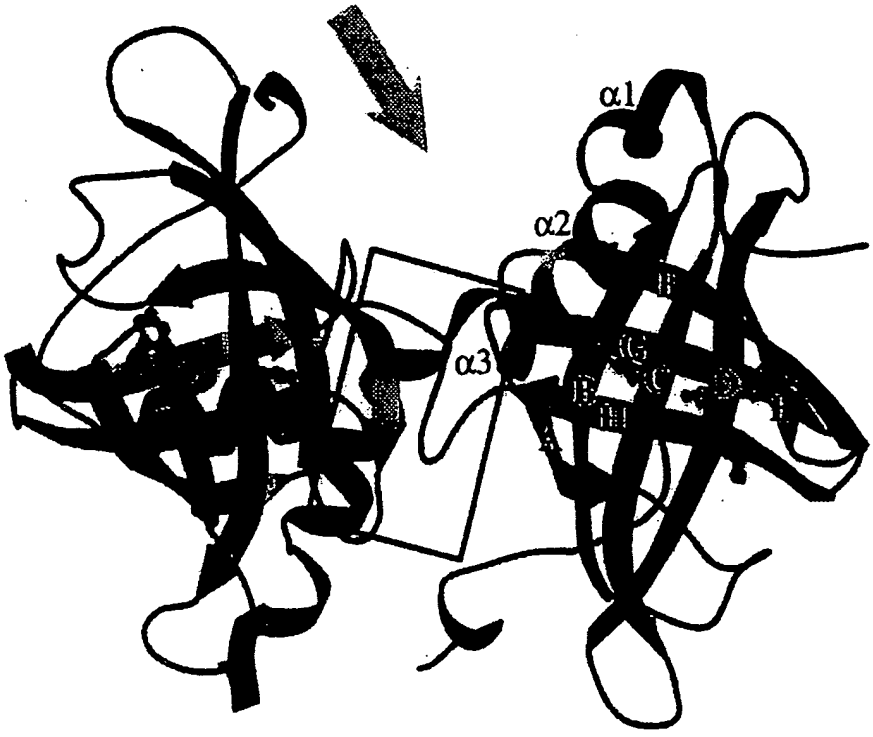


FIG. 20(b)

FIG. 20(c)

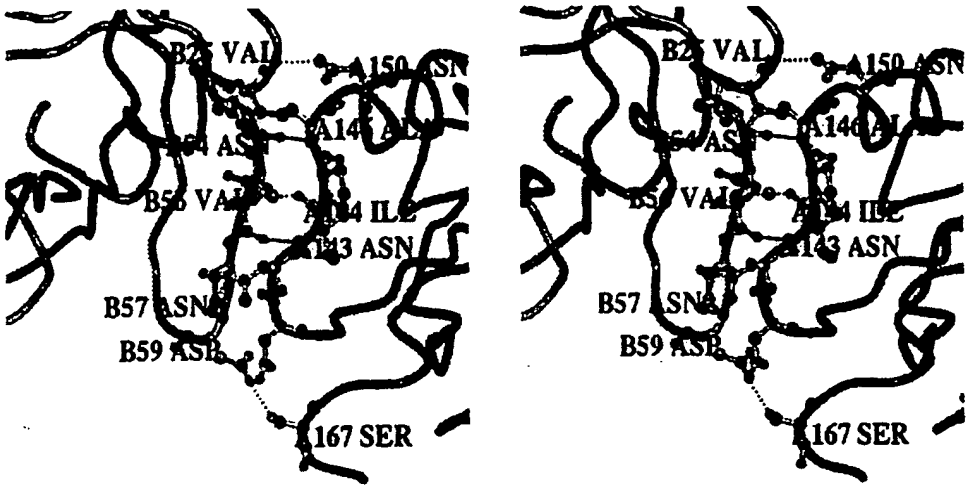


FIG. 20(d)

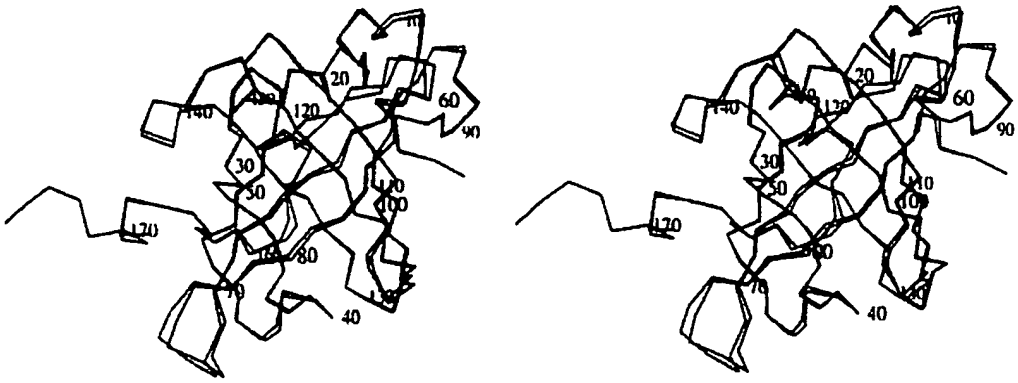


FIG. 21(a)

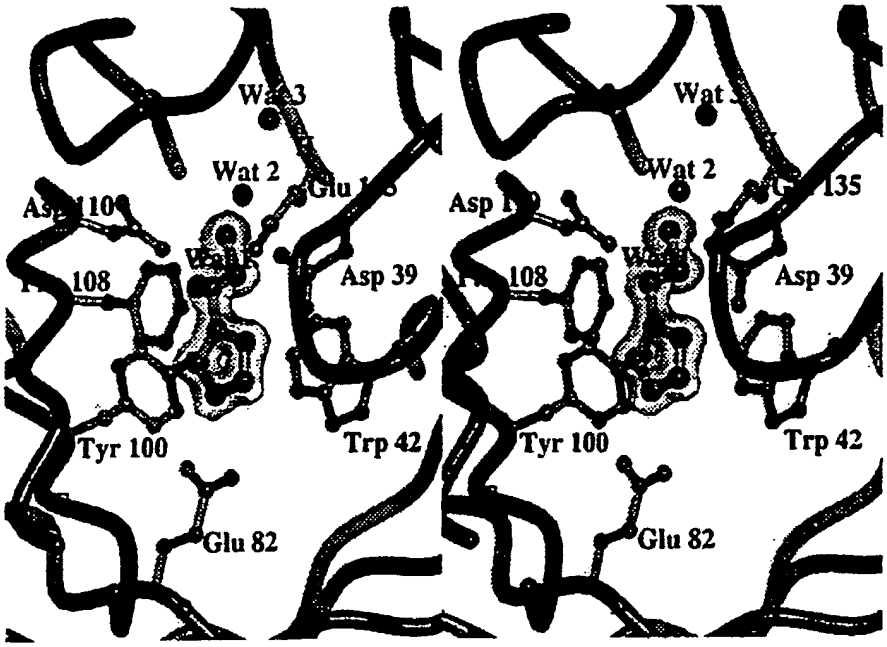


FIG. 21(b)

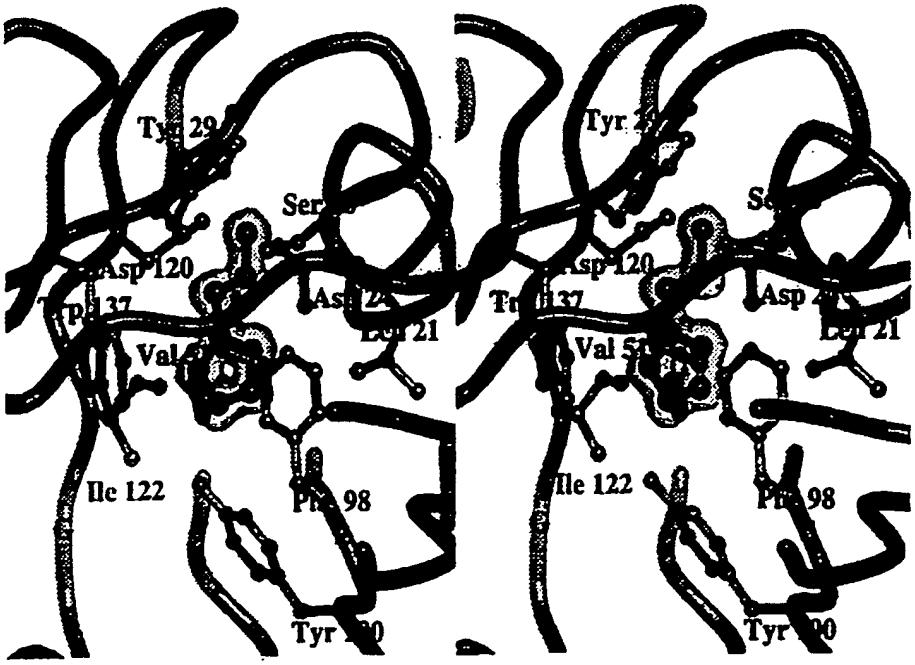
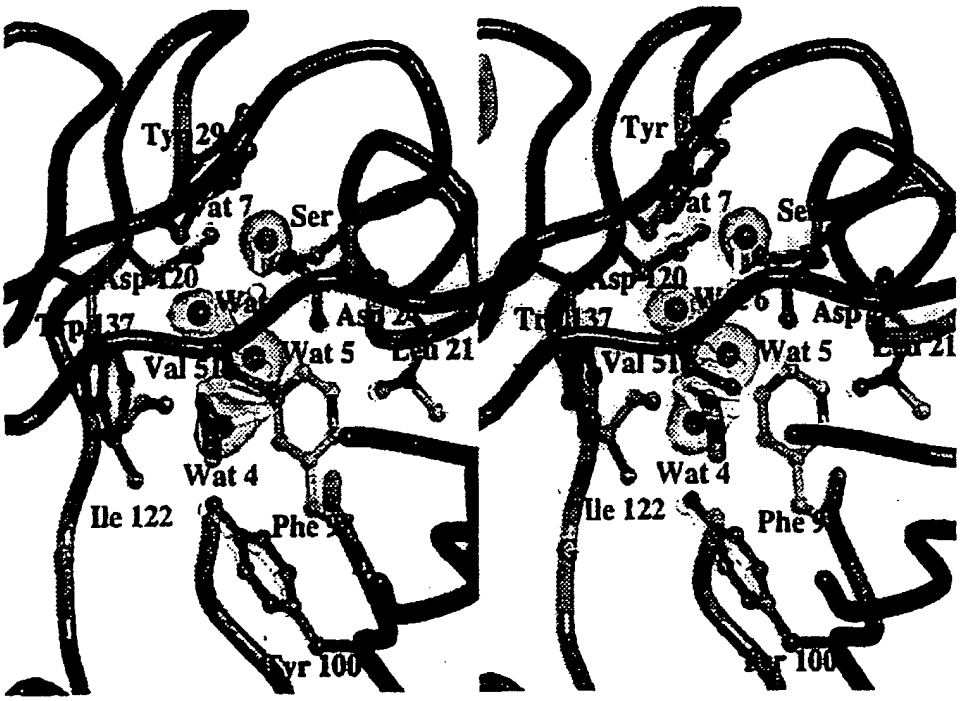


FIG. 21(c)


Tyr
Ser
Asp 120
Wat 5
Val 51
Wat 4
Ile 122
Tyr 100
Tyr
Ser
Wat 5
Val 51
Wat 4
Ile 122

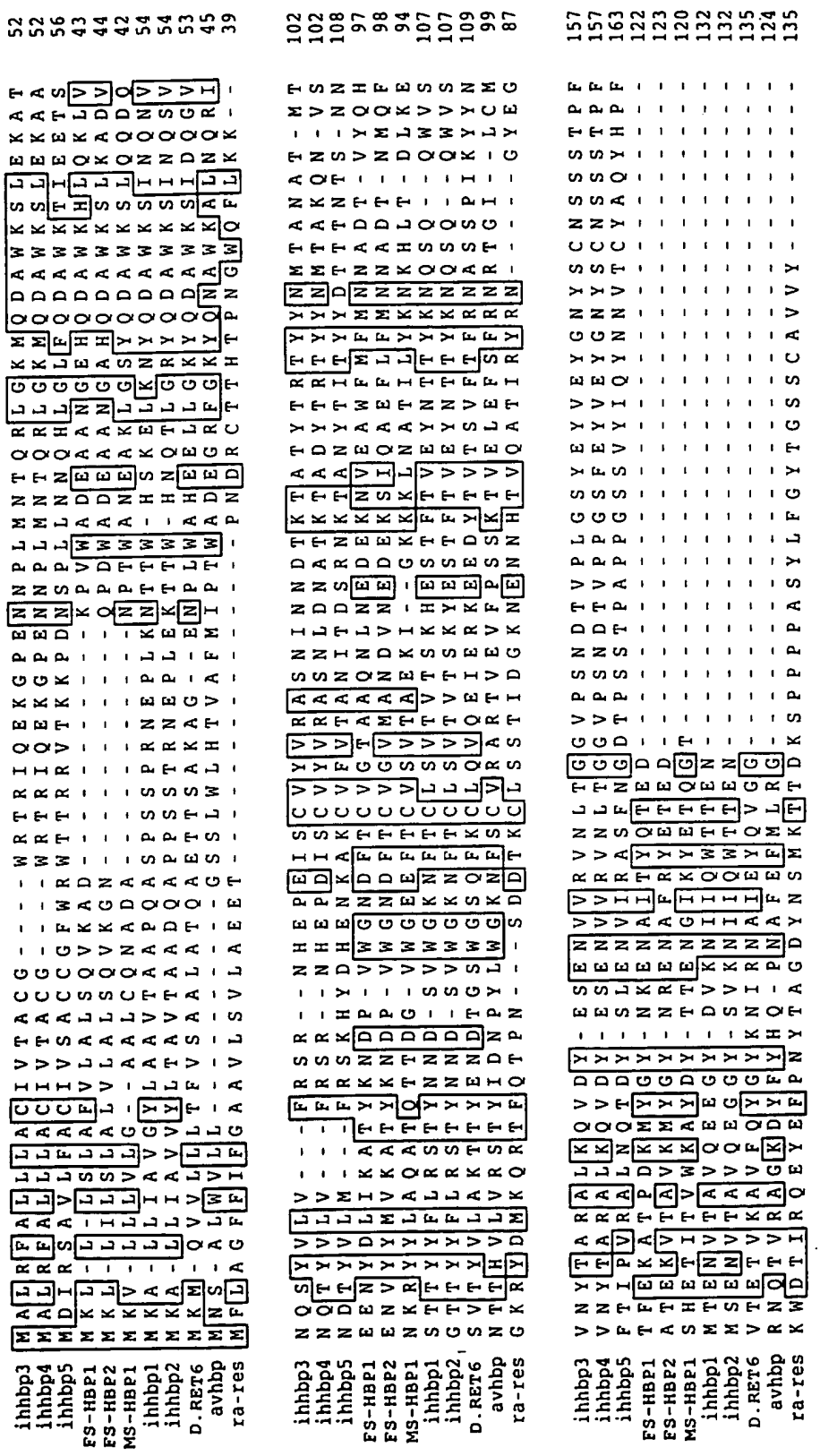


FIG. 22

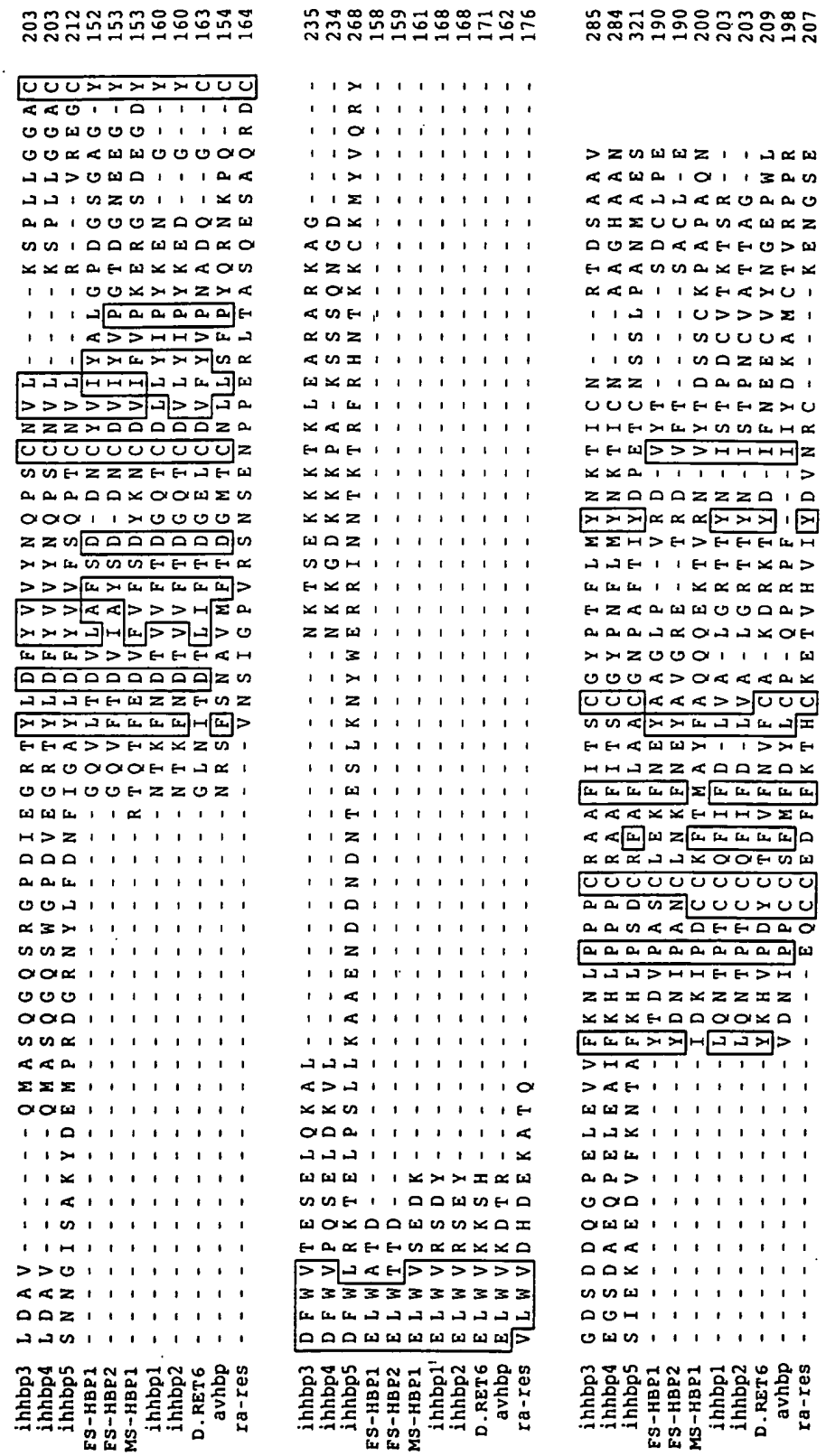


FIG. 22(CONTD.)

INTERNATIONAL SEARCH REPORT

Int .tional Application No
PCT/GB 98/03530

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/21 C07K14/435 A61K38/17 A61K31/40 A61K31/19
A61K31/66 A61K31/35 G01N33/68 A23L1/015 C12N5/10
C12N1/21 A01K67/027 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 44451 A (OXFORD VACS LTD ;PAESEN GUIDO CHRISTIAN (GB); NUTTALL PATRICIA ANN) 27 November 1997 see the whole document ---	1-7,10, 18-30, 33-50
A	RIBEIRO J M C ET AL: "HIGH AFFINITY HISTAMINE-BINDING AND ANTIHISTAMINIC ACTIVITY OF THE SALIVARY NITRIC OXIDE-CARRYING HEME PROTEIN (NITROPHORIN) OF RHODNIUS PROLIXUS" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 180, December 1994, pages 2251-2257, XP002050815 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 April 1999	27/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 98/03530

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WANG H ET AL: "COMPARISON OF THE PROTEINS IN SALIVARY GLANDS, SALIVA AND HAEMOLYMPH OF RHIPICEPHALUS APPENDICULATUS FEMALE TICKS DURING FEEDING" PARASITOLOGY, vol. 109, no. 4, November 1994, pages 517-523, XP002050816 ---	
A	WO 96 11271 A (HESKA CORP ;FRANK GLENN R (US); HUNTER SHIRLEY WU (US); WALLENFELS) 18 April 1996 ---	
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2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/GB 98/03530

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 36 and 38 are (partially) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/GB 98/03530

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